METHODS AND COMPOSITIONS FOR DETECTING AND TREATING RETINAL DISEASES

U.S. Utility patent application

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BACKGROUND OF THE INVENTION

Age-related macular degeneration (AMD) is the number one cause of blindness for the elderly population over 60 years of age. It is a devastating disease that destroys central vision in the affected individuals, robbing them of their ability to perform activities necessary for everyday life such as reading and driving (Bressler et al., 1988; Evans, 2001; Gottlieb, 2002). In one study, the prevalence of AMD in persons 75 or older has been reported to be 7.8% (Klein et al., 1992).

AMD is a slow, progressive disease that involves cells of the outer retinal layers (including photoreceptors and the retinal pigment epithelial (RPE) cells that support the photoreceptors), as well as cells in the adjacent vascular layer of the eye known as the choroid. Macular degeneration is characterized by the breakdown of the macula, a small portion of the central retina (about 2 mm in diameter) responsible for high-acuity vision. Late-onset macular degeneration (i.e., AMD) is generally defined as either "dry" or "wet." The wet ("exudative") neovascular form of AMD affects approximately 10% of those with the disease, and is characterized by abnormal blood vessels growing from the choriocapillaris through the RPE, typically resulting in hemorrhage, exudation, scarring, and/or serous retinal detachment. Approximately 90% of patients with AMD have the non-neovascular dry form, characterized by atrophy of the RPE and loss of macular photoreceptors.

One of the clinical hallmarks of AMD is the presence of deposits of debris-like material, termed "drusen," that accumulate on Bruch's membrane, a multilayered composite of extracellular matrix components separating the RPE (the outermost layer of the retina) from the underlying choroid. Drusen can be observed by funduscopic eye examination. These deposits have been extensively characterized in microscopic studies of donor eyes from patients with AMD (Sarks, et al., 1988). The deposits observed in the living eye upon clinical examination are classified as either soft drusen or hard drusen, according to several criteria including relative size, abundance, and shape of the deposits (reviewed, for example, in Abdelsalam et al., 1999). Histochemical and immunocytochemical studies have shown that drusen contain a variety of lipids,

polysaccharides, glycosaminoglycans and proteins (Abdelsalam et al., 1999; Hageman et al., 1999, 2001).

There is presently no cure for AMD. Several types of treatments are available, with laser photocoagulation of abnormal vessels in the wet form of the disease being the standard (Gottlieb, 2002; Algvere and Seregard, 2002). This treatment is limited by the fact that only well-delineated neovascular lesions can be treated in this way and that 50% of patients will suffer recurrence of the leakage from the vessels (Fine et al., 2000). Because of the energy of the laser required for this treatment, the photoreceptors in the treated area will also die, and the patient will also often suffer central blindness immediately after the treatment. New neovascular lesions will eventually develop, requiring repeated treatments.

Photodynamic therapy, which combines low energy laser activation with a photosensitive agent, has been a valuable addition to the laser treatment approach (Bressler, 2001). In this method, a photosensitive agent, i.e., verteporfin is used which has an affinity for abnormal new blood vessels. Selective targeting of these vessels can be activated by nonthermal laser to produce reactive oxygen species which can destroy the abnormal vessels. In a study group, only 33% of those receiving photodynamic therapy with verteporfin had substantial loss of vision, compared to 61% of those who did not receive verteporfin. The treatment, however, was only beneficial for patients with classic choroidal neovascular membranes. The full long-term benefit of this new treatment modality has yet to be established. Despite this advance, however, the treatment does not prevent the subsequent formation of new neovascular lesions.

Other available treatments for the wet form of AMD include submacular surgery and external-beam radiation therapy. Those under study include retinal translocation and inhibition of vascular endothelial growth factor (Algvere and Seregard, 2002). For prevention of progression to advanced AMD, treatment with antioxidants, including vitamins C and E, β-carotene, and zinc, was shown to be helpful, and prophylactic laser treatment is under study (Gottlieb, 2002).

Despite the above-described advances, it is recognized that current treatment for AMD is mostly palliative (Algvere and Seregard, 2002). None of the available treatments attacks the fundamental cause of the disease, which is unknown. The disease

therefore can continue to progress following treatment, with re-development of neovascularization and destruction of the macula. Accordingly, there remains a compelling need to understand the molecular mechanism of this disease, so that therapeutic treatment or cure can be directed at its root cause.

It is well recognized that genetic factors play an important role in the etiology of AMD. For example, it has been reported that people with a family history of AMD and siblings of AMD patients have a higher risk of developing AMD (Evans, 2001). Monozygotic twins have shown a higher concordance rate of clinical features of AMD compared to dizygotic twins (Klein et al., 1994). Another study found all monozygotic twins affected with AMD to be concordant for AMD while only 42% of dizygotic twins were concordant (Meyers et al., 1995). Accordingly, one major approach to understanding AMD etiology is to look for genes involved in AMD. For example, approaches such as linkage analysis in large families, allele sharing analysis among sib pairs, and association studies in populations have been used in attempts to identify genes associated with AMD (Guymer, 2001). Linkage to chromosomal region 1q was reported in a large AMD family (Klein et al., 1998). Results of an allele sharing analysis did not yield any new candidate genes (Weeks et al., 2000). An association of a mutation in hemicentin-1 has been reported in a familial form of age-related macular degeneration mapping to human chromosome 1q in a large family (Schultz et al., 2003).

Another genetic strategy for AMD is to test genes causing other forms of inherited macular degenerations as putative causative genes ("candidate genes") for AMD. Several macular diseases with a clearly hereditary pattern of inheritance (so-called "Mendelian macular degenerations") have been described that resemble AMD in phenotype. Examples of these diseases include Sorsby's fundus dystrophy, Stargardt's disease, Best disease, and Doyne's honeycomb retinal dystrophy (Guymer, 2001). Causative genes for these diseases have been analyzed as candidate genes for AMD. To date however, none of them has clearly demonstrated a causal relationship with AMD. For example, the ATP-binding cassette transporter gene (ABCR) was found to be the pathogenic gene for recessive Stargardt's disease (Hutchinson et al., 1997). ABCR was proposed as a candidate gene for AMD, and in one study, 16% of patients with AMD

were initially shown to have mutations in this gene (Allikmets et al., 1997). This conclusion, however, has been challenged (Stone et al., 1998).

The most likely reason for the failure to find AMD genes through classical genetic approaches such as chromosomal mapping, genetic linkage analysis, and candidate gene analysis, is that AMD is a "multigene,"or "complex" genetic disease. Complex genetic diseases are those diseases believed to be caused by changes in multiple genes. Such diseases characteristically demonstrate a complex pattern of inheritance (Heiba et al., 1994; Klein et al., 1994). In the case of AMD, a disease of old age, it is generally thought that the course of the disease is influenced not only by the combined effects of the above-described multiple genetic factors, but is further affected by certain environmental risk factors.

A second broad approach aimed at discovering causative genes in AMD has been hypothesis-based research aimed at elucidating the mechanism of the disease, with the goal of secondarily identifying the genes involved in the mechanism. Numerous hypotheses regarding the pathogenic mechanism of AMD have been proposed and tested, resulting in a voluminous literature on this subject.

Oxidative damage has been one major theme as a proposed mechanism for AMD (Winkler et al., 1999; Evans, 2001; Husain et al., 2002). The retina is known to have an extremely high consumption of oxygen, and the photoreceptors and RPE are in a very oxygen-rich environment. The RPE is situated immediately adjacent to the choriocapillaris, a rich capillary plexus coursing with highly oxygenated blood. The retina is a light-sensitive organ in which photoactivated events are constantly occurring during times of light exposure, resulting *inter alia* in the production of reactive oxygen species. In general support of the oxidative damage hypothesis, antioxidants tested in clinical studies have been reported to have a moderate beneficial effect of reducing progression to severe AMD (Hyman and Neborsky, 2002), although the results of several studies are conflicting (Flood et al., 2002). Smoking, which can reduce plasma levels of antioxidants, has been associated with increased risk of AMD (Mitchell et al., 2002). Adding support to the oxidative damage theory is a recent proteomic analysis of drusen, which demonstrated the presence in these deposits of several oxidation-modified products (Crabb et al., 2002).

It has been proposed that dysfunction in the RPE is central to the pathogenesis of AMD and can lead to drusen formation (Hogan, 1972). The earliest known sign of RPE dysfunction is accumulation of lipofuscin, which may lead to the characteristic thickening of Bruch's membrane, formation of drusen, and choroidal neovascularization observed in the wet form of AMD (Gass et al., 1985; Sarks et al., 1988; Green, 1999). Lipofuscin is composed of oxidized, polymeric molecules derived mostly from phagocytosed membranes of photoreceoptor outer segments (OS) (Katz, 1989; Kennedy et al., 1995). OS membranes are known to be rich in polyunsaturated fatty acids, which are an excellent substrate for peroxidation (Katz, 1989). It is believed that these molecules cannot be degraded and therefore begin to accumulate in the RPE cells as lipofuscin. At least one component of lipofuscin, i.e., the fluorophore A2E, a pyridinium bisretinoid, has been demonstrated to be toxic, causing membrane destabilization (De and Sakmar, 2002), and inhibition of cytochrome c oxidase and apoptosis in cultured porcine and human RPE cells (Shaban et al., 2002). Thus, A2E and lipofuscin accumulation in the RPE is thought to be directly related to dysfunction and demise of these cells with aging.

The processes of oxidative damage, lipofuscin accumulation, and drusen formation are not limited to AMD, but rather occur to some extent in all individuals with advancing age. Accordingly, a fundamental question that remains unanswered is why these processes are more advanced in some people than others, leading to AMD. Progress in developing new therapies targeting the root cause of AMD will require much greater knowledge of specific gene targets involved in the key cellular metabolic pathways in photoreceptors, RPE and choroidal cells that contribute to the observed pathology.

SUMMARY OF THE INVENTION

The invention provides novel methods and compositions for screening and treating retinal degenerative conditions, including age-related macular degeneration (AMD), as well as animal models useful for testing therapeutic compounds and methods. The invention is the product of a gene discovery strategy resulting in isolation of genes showing differential expression 1) in AMD-affected vs. normal eye tissues and 2) during the process of phagocytosis of outer segments (OS) by RPE cells. OS phagocytosis is a critical function of the RPE cells, involving a complex multi-step process, the byproducts of which contribute to generation of reactive oxygen species and lipofuscin accumulation in the RPE cells.

Using a novel expression cloning strategy termed CHANGE (for Comparative Hybridization ANalysis of Gene Expression) at least 200 AMD-related genes and at least 60 phagocytosis-related genes expressed in RPE cells were isolated. Five previously uncharacterized genes were identified by this strategy and demonstrated to be related to AMD and/or RPE phagocytosis. The nucleic acid sequences of cDNAs encoding the products of these genes are listed herein as SEQ ID NOS:1, 4, 5, 12, and 17.

A subset of six genes, termed "AMD/phagogenes," or "AMDP genes" are further described herein that fit the dual criteria of relatedness to AMD and to RPE phagocytosis. Three of these genes, i.e., prostaglandin D2 synthase (SEQ ID NO:2), matrix metalloproteinase, membrane-type 1 (MT1-MMP) (SEQ ID NO:15), and unknown RPE-expressed cDNA AMDP-3 (SEQ ID NO:17) all demonstrate up-regulation in AMD. AMDP genes down-regulated in AMD include casein kinase 1 epsilon (SEQ ID NO:9), ferritin heavy polypeptide 1 (SEQ ID NO:10), and SWI/SNF related/OSA-1 nuclear protein (SEQ ID NO:16).

Other genes previously not known to be functionally related to RPE phagocytosis are disclosed herein, including unknown PHG-1 (SEQ ID NO:1), myelin basic protein (SEQ ID NO:3), unknown PHG-4 (SEQ ID NO:4), unknown PHG-5 (SEQ ID NO:5), peanut-like2/septin 4 (SEQ ID NO:6), coactosin-like 1 (SEQ ID NO:7), clusterin (SEQ ID NO:8), metargidin (SEQ ID NO:11), unknown PHG-13 (SEQ ID NO:12), retinaldehyde binding protein 1 (SEQ ID NO:13), and actin gamma 1 (SEQ ID NO:14).

An exemplary AMDP gene discovered by the above strategy is the membrane-type matrix metalloproteinase 1 (MT1-MMP) (SEQ ID NO:15). MT1-MMP is a gene encoding a protease involved in the remodeling of extracellular matrix, for example by specifically activating pro-gelatinase A. Gelatinase A is the major metalloproteinase responsible for specific cleavage of type IV collagen, the main structural component of basement membranes. MT1-MMP also shows activity against other extracellular matrix components.

It has been demonstrated that MT1-MMP is a highly attractive therapeutic target for screening and treating AMD and other retinal conditions, based on the following findings:

1) MT1-MMP is upregulated in the RPE and photoreceptors in the eyes of patients with AMD, in a monkey model of AMD, and in the RCS rat, a model of retinal degeneration involving a defect in OS phagocytosis by the RPE;

2) MT1-MMP is directly involved in the mechanism of phagocytosis by RPE cells;

3) the progress of retinal degeneration in the RCS rat is significantly reduced by blocking activated MT1-MMP present in the subretinal space with an anti-MT1-MMP antibody;

4) a synonymous polymorphism of MT1-MMP (i.e., P259P) that could produce a splice variant of the mRNA resulting in a truncated protein, and a missense polymorphism of MT1-MMP (i.e., D273N) affecting the catalytic domain of the protein are found with higher frequency in the DNA of patients with AMD (54.8% vs. 31.6%) and familial maculopathies (68.2% vs. 31.6%).

Based on the foregoing discoveries, it is an object of the invention to provide a method for delaying or reversing a retinal or choroidal degenerative disease or condition in a subject. The method includes contacting a retinal or choridal cell of a subject having, or at risk of developing, a retinal or choroidal degenerative disease or condition with an agent that modulates the expression or activity of an AMDP-related or phagocytosis-related gene. The AMDP-related or phagocytosis-related gene can be human unknown PHG-1; prostaglandin D2 synthase; myelin basic protein; human unknown PHG-4; human unknown PHG-5; human peanut-like 2/septin 4; coactosin-like 1; clusterin; casein kinase 1 epsilon; ferritin heavy polypeptide 1; metargidin; human unknown PHG-13; retinaldehyde binding protein 1; actin gamma 1; matrix metalloproteinase, membrane-

associated 1 (MT1-MMP); SWI/SNF related/OSA-1 nuclear protein; and human unknown AMDP-3. The foregoing AMDP-related or phagocytosis-related genes include, respectively, the nucleotide sequences identified herein as SEQ ID NOS:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 17.

Preferred genes targeted for modulation of expression or activity are prostaglandin D2 synthase, MT1-MMP and unknown gene AMDP-3, shown herein to be up-regulated in AMD. In a particularly preferred embodiment, the agent is directed against a MT1-MMP nucleic acid or protein. The retinal or choroidal degenerative disease or condition can be AMD. The method can be used to treat a subject suffering from AMD, or at risk of developing AMD.

The method can delay the retinal or choroidal degenerative disease or condition, or it can reverse the disease or condition.

The cell type to be contacted in the practice of the method can be a photoreceptor, an RPE cell or a Muller cell, or a cell type of the choroid, including an endothelial cell, a smooth muscle cell, a leukocyte, a macrophage, a melanocyte or a fibroblast.

In a preferred embodiment of the method, in which the AMDP-related or phagocytosis-related gene is MT1-MMP,the MT1-MMP may be located within the cell or in an extracellular matrix, such as an interphotoreceptor matrix.

In some embodiments of the method, the agent down-regulates expression of a nucleic acid or amino acid sequence of an AMDP-related or phagocytosis-related gene. In preferred embodiments, the targeted genes include MT1-MMP, prostaglandin D2 synthase and AMDP-3, which genes are shown herein to be over-expressed in AMD. The agent may be an oligonucleotide, for example a ribozyme, an antisense RNA, an interfering RNA (RNAi) molecule, or a triple helix forming molecule.

The agent may also be an antibody that specifically binds to a MT1-MMP, prostaglandin D2 synthase or AMDP-3 protein or peptide. Preferably the antibody can neutralize at least one biological activity of the protein or peptide. For example, an antibody against MT1-MMP can neutralize activation of a progelatinase A, or degradation of an extracellular matrix component.

In another embodiment, the agent that down-regulates expression of MT1-MMP, prostaglandin D2 synthase or AMDP-3 can be a small molecule.

It is a further object of the invention to provide a method of determining risk of a subject of developing a retinal or choroidal degenerative disease or condition. The method includes screening a nucleic acid sequence of the subject for the presence of at least one polymorphism in at least one phagocytosis-related or AMDP-related gene, wherein the presence of a polymorphism indicates that the subject is at higher risk for developing a retinal degenerative disorder than a subject without the polymorphism.

The phagocytosis-related genes can include, but are not limited to, unknown PHG-1, prostaglandin D2 synthase, myelin basic protein, unknown PHG-4, unknown PHG-5, peanut-like 2/septin 4, coactosin-like 1, clusterin, casein kinase 1 epsilon, ferritin heavy polypeptide 1, metargidin, unknown PHG-13, retinaldehyde binding protein 1, actin gamma 1, membrane type metalloprotinase 1 (MT1-MMP), SWI/SNF related/OSA-1 nuclear protein, and unknown AMDP-3. Nucleic acids encoding these phagocytosis-related gene products include, respectively, cDNA sequences listed herein as SEQ ID NOS:1-17.

The AMDP-related genes to be screened in the method can include, but are not limited to, prostaglandin D2 synthase, casein kinase 1 epsilon, ferritin heavy polypeptide 1, SWI/SNF related/OSA-1 nuclear protein, and AMDP-3. Nucleic acids encoding these AMDP-related gene products include, respectively, cDNA sequences listed herein as SEQ ID NOS:2, 9, 10, 16 and 17.

The polymorphisms screened in the method can be within an intronic, exonic or promoter region of the gene of interest.

In a preferred embodiment of the screening method, the gene of interest is MT1-MMP. The polymorphism can be within a region of the human MT1-MMP gene that can be amplified by PCR using amplimer pairs having nucleic acid sequences selected from the following groups: SEQ ID NOS:18 and 19; 20 and 21; 22 and 23; 24 and 25; 26 and 27; 28 and 29; 30 and 31; 32 and 33; 34 and 35; 36 and 37; 38 and 39; 40 and 41; 42 and 43; 44 and 45; 46 and 47; 48 and 49; 50 and 51; 52 and 53; 54 and 55; 56 and 57; and 57 and 58.

In a particularly preferred embodiment of the method, the polymorphism is within a 285 bp fragment of exon 5 of the human MT1-MMP gene. Within this region, the

polymorphisms can include a D273N missense polymorphism and a P259P synonymous polymorphism.

It is also an object of the invention to provide a method of treating a retinal or choroidal degenerative disease or condition in a subject. The method includes contacting a retinal or choroidal cell of the subject with a vector that includes a nucleic acid encoding an agent that down-regulates or inhibits expresion of a phagocytosis-related or AMDP-related mRNA or protein. The agent included in the vector can be an antisense RNA, a ribozyme, or an interfering RNA (RNAi) molecule. In preferred embodiments, the phagocytosis-related or AMDP-related genes targeted for down-regulation are prostaglandin D2 synthase, MT1-MMP, and AMDP-3, comprising respectively the nucleic acid sequences shown herein as SEQ ID NOS:2, 15 and 17.

In another aspect, the invention provides a method of treating a retinal or choridal degenerative disease or condition using a vector to deliver a desired form of a phagocytosis-related or AMDP-related gene product to a subject in need thereof. The vector can include a nucleic acid encoding either a wild type or polymorphic variant of a phagocytosis-related or AMDP-related gene.

Yet another embodiment of the invention is a composition for prevention or treatment of a retinal or choroidal degenerative disease or condition in a subject comprising an agent that blocks the expression or activity of a phagocytosis-related or AMDP-related gene. In some embodiments, the agent can be an antisense RNA, a ribozyme, or an interfering RNA (RNAi) molecule. The agent can also be an antibody or a small molecule.

Also within the invention are compositions for prevention or treatment of a retinal or choroidal degenerative disease or condition in a subject comprising a vector. In various embodiments, the vectors can include a nucleic acid encoding an agent that down-regulates or inhibits expression of a phagocytosis-related or AMDP-related mRNA or protein, or a nucleic acid that encodes a wild type or polymorphic variant of a phagocytosis-related or AMDP-related protein. In preferred embodiments, the phagocytosis-related or AMDP-related genes include MT1-MMP, prostaglandin D2 synthase and AMDP-3. In particularly preferred embodiments, the gene is MT1-MMP.

The invention further provides several embodiments of nonhuman transgenic animals useful, for example, as models of AMD and other retinal degenerative conditions. Preferably, the transgenic animal is a mammal, more preferably a rodent, and most preferably a mouse. In one embodiment, a transgenic animal includes an isolated nucleic acid construct that causes at least one cell type of the animal to over-express a phagocytosis-related or AMDP-related gene. The phagocytosis-related or AMDP-related gene is preferably MT1-MMP, prostaglandin D2 synthase, or AMDP-3. Preferred versions of the transgenic animals are engineered to overexpress the phagocytosis-related or AMDP-related gene product in particular cell types, including retinal cell types selected from photoreceptors, RPE cells and Muller cells, and choroidal cell types including endothelial cells, smooth muscle cells, leukocytes, macrophages, melanocytes and fibroblasts. In some embodiments, the gene of interest is conditionally over-expressed.

Another preferred embodiment of an animal model of AMD/retinal degeneration is a nonhuman transgenic animal including an isolated nucleic acid construct that causes at least one cell type of the animal to express a polymorphic variant of a phagocytosis-related or AMDP-related nucleic acid and/or protein. In preferred embodiments, the nucleic acid and/or protein is MT1-MMP, prostaglandin D2 synthase, or AMDP-3. The polymorphic variant can be increased in incidence in a population of humans with AMD, compared to a normal control population.

Yet another embodiment is a nonhuman polytransgenic animal including at least a first isolated nucleic acid construct and at least a second isolated nucleic acid construct, the first construct causing at least one cell type of the animal to express a polymorphic variant of a first gene correlated with increased incidence of AMD, and the second nucleic acid construct causing at least one cell type of the animal to express a polymorphic variant of a second gene correlated with increased incidence of AMD, or havingan association with RPE phagocytosis.

In preferred embodiments of the polytransgenic animals, the first gene is MT1-MMP and the second gene is selected from ABCR, apolipoprotein E, C-C chemokine receptor-2, cystatin C, hemicentin/FIBL-6, manganese superoxide dismutase, C-C chemokine ligand/monocyte chemoattractant protein 1, and paraoxonase.

In other preferred embodiments of the polytransgenic models, the first gene is MT1-MMP and the second gene is a phagocytosis-related or AMDP-related gene selected from human unknown PHG-1, prostaglandin D2 synthase, myelin basic protein, human unknown PHG-4, human unknown PHG-5, human peanut-like 2/septin 4, coactosin-like 1, clusterin, casein kinase 1 epsilon, ferritin heavy polypeptide 1, metargidin, human unknown PHG-13, retinaldehyde binding protein 1, actin gamma 1, SWI/SNF related/OSA-1 nuclear protein, and human unknown AMDP-3.

Particularly preferred embodiments of the transgenic animals of the invention are mice, which provide the advantage of a relatively short life span, making them more suitable for study of age-related diseases than other longer-lived animal models such as monkeys.

In yet another aspect, the invention provides isolated nucleic acids encoding previously uncharacterized gene products shown herein to be phagocytosis-related and/or AMDP-related proteins. The nucleic acids encoding these proteins include nucleic acid sequences comprising SEQ ID NOS:1, 4, 5, 12, and 17.

The invention further provides a gene array including a plurality of isolated oligonucleotide sequences, said sequences being positioned within an intronic, exonic or promoter sequence of a native human AMD-related or phagocytosis-related gene. The genes represented by the oligonucleotide sequences in the array encode cDNAs comprising nucleic acid sequences shown herein as SEQ ID NOS:1-17 and SEQ ID NOS:62-69.

In preferred embodiments of the gene array, at least one gene is MT1-MMP and the oligonucleotide sequences include a P259P or a D273N polymorphic variant of the MT1-MMP coding sequence. These variants of MT1-MMP are shown herein to be increased in frequency in a population of patients with AMD and other macular degenerative conditions, relative to their frequency in a population of normal control subjects.

The gene array can further include at least one oligonucleotide sequence comprising at least one polymorphic variant of one or more AMD-related genes besides MT1-MMP. The polymorphic variant sequences can include:ABCR (D217N; G1961E),

manganese superoxide dismutase (V47A), apolipoprotein E (C130, R176C and C130R, R176), cystatin C (A25T) and paraoxonase (Q192R, L54M).

The gene arrays of the invention are useful, for example, for screening DNA samples from subjects to determine the distribution of polymorphic variants of a plurality of AMD-related and/or phagocytosis-related genes in the subject's DNA. In keeping with the multi-gene (complex disease) etiology of AMD, it is contemplated that information pertaining to the distribution of combinations of particular polymorphic variants of AMD-related or phagocytosis-related genes in a subject's DNA can be used to predict the likelihood that the subject is at greater risk of developing a retinal disorder such as AMD than is a subject lacking said combination of particular polymorphic variants of AMD-related or phagocytosis-related genes.

The gene arrays of the invention, tailored to AMD and related disorders, can provide a convenient and relatively inexpensive means of testing polymorphic variants of a plurality of genes known to be related to AMD and related disorders.

These and other objects of the invention are set forth in more detail in the description and examples below, which are intended to illustrate the invention but not limit the scope thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of the following drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 is a photograph showing duplicate CHANGE array panels, each containing 96 genes (spots) hybridized with "+" and "-" probes (Probes 1 and 2), according to an embodiment of the invention. Up and down arrows indicate genes showing increased or decreased expression, respectively, upon hybridization with Probe 1 vs. Probe 2.

FIG. 2 (upper panel) shows a schematic drawing of a vital assay of rod outer segment (ROS) phagocytosis by cultured RPE cells. The lower panel shows black and white photographs of living BPEI-1 RPE cells undergoing phagocytosis after ROS feeding, according to an embodiment of the invention. When observed by fluorescence microscopy, lysosomes in the RPE cells appear red due to sulforhodamine (SR) staining and FITC-stained ROS appear green. During successive stages of phagocytosis, ROS are bound to the cell surfaces, then ingested by the RPE cells, first becoming phagosomes and then phagolysosomes (distinguishable by yellow-orange fluorescence) upon fusion with lysosomes.

FIG. 3 is a series of photographs showing different stages of ROS phagocytosis viewed in large scale cultures of living BPEI-1 RPE cells at the indicated times after feeding with FITC-ROS, according to an embodiment of the invention. The upper four panels show massive binding of ROS to the cell surfaces during the first 9-10 hours after feeding. The lower four panels show synchronous ROS ingestion and formation of phagolysosomes, starting approximately 11 hours after feeding with ROS.

FIG. 4 is a graph showing the mRNA expression profiles of 16 phagocytosis-related genes ("phagogenes") expressed by RPE cells, discovered by CHANGE, according to an embodiment of the invention. Expression levels of phagogenes fluctuate in RPE cells at selected times during the course of ROS phagocytosis *in vitro*. Identities of the phagogenes (PHG-1-16) are provided in Table 1, *infra* .

FIG. 5 is three photographs showing the grading system used to classify human donor eyes for AMD-related changes in the retina, according to an embodiment of the

invention. Grades shown: 0 - +1, minimal thickening of the Bruch's membrane; +2 - +3, multiple small to mid size drusen, with thickened Bruch's membrane; +3 - +4, large coalescing drusen.

FIG. 6 is a two Northern blots and a graph showing expression of MT1-MMP and actin mRNA during phagocytosis by cultured RPE cells at 4 and 13 hours after ROS feeding. Decreased expression at 4 hours and increased expression at 13 hours is seen, confirming results obtained by CHANGE. The amount of RNA present in each lane is estimated by actin hybridization, used to normalize the MT1-MMP hybridization signal.

FIG. 7 is a graph showing a fluctuating (diurnal) pattern of expression of MT1-MMP mRNA in the normal rat retina, according to an embodiment of the invention. The highest level of MT1-MMP expression occurs at 6 AM, approximately 1-2 hours before the time of maximal shedding and phagocytosis of the photoreceptor (OS) *in vivo*.

FIG. 8 is eight photomicrographs (phase contrast and fluorescence) showing immunofluorescent staining of normal rat retina fixed at various times of day and immunostained with an anti-MT1-MMP antibody, according to an embodiment of the invention. Diurnal variation is seen in the immunofluorescence level of MT1-MMP protein present in the OS and RPE, with the highest level of signal observed at 6 AM, less at 10 AM, and no signal at 10 PM, consistent with the diurnal pattern of MT1-MMP mRNA expression levels shown in FIG. 7.

FIG. 9 is a fluorescence micrograph of a section of human retina stained with anti-MT1-MMP antibody, showing localization of the MT1-MMP protein in the OS of rod and cone photoreceptors and in phagosomes within the RPE cells, according to an embodiment of the invention.

FIG. 10 (A-C) is three fluorescence micrographs showing the effect of anti-MT1-MMP antibody on ROS phagocytosis by RPE cells in culture, according to an embodiment of the invention. Ingestion of the fed ROS (fluorescence) is evident in the cytoplasm in control cells not incubated with antibody (B) and in cells incubated with an unrelated (X-arrestin) antibody (C), whereas ROS binding and phagocytosis does not occur in cells incubated with anti-MT1-MMP antibody prior to feeding with ROS (A).

FIG. 11 (A-D) is four micrographs of H&E stained paraffin sections of normal rat retina showing the effect of subretinal injection of anti-MT1-MMP antibody on the

structure of the outer retina. Pronounced lengthening and abnormal orientation of the OS, consistent with inhibited OS phagocytosis, is observed in the anti-MT1-MMP antibody injected left eye, O.S. (A, B). In contrast, retinal architecture is normal in the uninjected right eye (O.D.) of the same animal (C). Subretinal injection of an unrelated (X-arrestin) antibody has no effect (D).

FIG. 12 shows Northern blot analysis of MT1-MMP mRNA expression levels in the RPE/choroid and retina of a subject affected with AMD (A) compared to a normal control subject (N). A 5.5-fold increase in the level of MT1-MMP mRNA is seen in the affected retina, with a 1.2-fold increase in the RPE/choroid of this subject. The Northern blot hybridization signals are normalized with respect to the amount of RNA present in each lane using actin hybridization as a reference.

FIG. 13 is a graph showing a positive correlation of level of expression of MT1-MMP mRNA with increasing severity of AMD-related pathology (grade 0 – +4 changes) in retinas of subjects affected with AMD.

FIG. 14 shows the nucleic acid sequence of a 285 bp PCR product including exon 5 of human MT1-MMP. The positions of codons 259 and 273 are underlined. Bases showing changes in polymorphisms P259P and D273N found in AMD and macular degeneration patients are indicated in boldface.

FIG. 15 is two micrographs showing a delay in inherited retinal degeneration in an RCS rat injected subretinally on postnatal day 7 with an anti-MT1-MMP antibody and fixed at 30 days of age. The delay in retinal degeneration is evidenced by the greater number of photoreceptor nuclei (approximately double) remaining in the outer nuclear layer of the retina of the injected eye (A), compared to a comparable mid-central region in the uninjected control eye of the same animal (B).

DETAILED DESCRIPTION OF THE INVENTION

Based on the foregoing discoveries, the invention provides novel genes related to AMD and/or phagocytosis by RPE cells, methods and compositions for detecting and treating AMD and other retinal degenerative conditions, and animal models based on phagocytosis-related and/or AMDP-related genes useful, *inter alia*, for testing therapeutic compounds and treatment protocols for AMD. The below described preferred embodiments illustrate adaptations of these compositions and methods. Nonetheless, from the description of these embodiments, other aspects of the invention can be made and/or practiced based on the description provided below.

Biological Methods

Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Various techniques using polymerase chain reaction (PCR) are described, for example, in Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Carruthers, Tetra. Letts. 22:1859-1862, 1981, and Matteucci et al., J. Am. Chem. Soc. 103:3185, 1981. Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers. Immunological methods (for example, preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, for example, in Current Protocols in Immunology, ed. Coligan et al., John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff et al., John Wiley & Sons, New York, 1992. Conventional methods of gene transfer and gene therapy can also be adapted for use in the present invention. See, for example, Gene Therapy: Principles and Applications, ed. T. Blackenstein, Springer Verlag, 1999; Gene Therapy Protocols (Methods in Molecular Medicine), ed. P.D. Robbins, Humana Press, 1997; and Retro-vectors for Human Gene Therapy, ed. C.P. Hodgson, Springer Verlag, 1996.

Phagocytosis-Related Genes Isolated by CHANGE

Studies leading to the invention were performed to identify genes involved in OS phagocytosis by RPE cells that, when perturbed, could result in stress and dysfunction in the RPE. Such stresses could lead to one or more undesirable changes associated with macular, retinal or choroidal diseases, such as enhanced lipofuscin accumulation, drusen formation, or formation of neovascular membranes. The gene discoveries described herein were based on the premise that dysfunction in phagocytosis by the RPE is a key factor leading to such AMD-related changes. RPE cells perform the crucial function of sustaining the homeostasis of the photoreceptors. This demanding task includes *inter alia* a daily process of phagocytosis and digestion of OS membranes which are renewed and shed daily from the tips of the OS of the photoreceptors (Young and Bok, 1969). As further described below, the phagocytic process includes the steps of binding, ingestion and digestion of OS membranes. Under normal circumstances, RPE cells are non-dividing cells. Thus, throughout the lifetime of an individual, the daily process of OS phagocytosis represents not only an enormous metabolic load on these cells, but also contributes to the accumulation within these cells of undigested material, particularly lipofuscin, a complex amalgam of cellular waste products including toxic photoreceptor-derived materials such as A2E.

Accordingly, in one aspect, the invention provides nucleic acid and protein sequences of genes previously unknown to be functionally related to the process of phagocytosis by RPE cells. Prior to the invention, there had not been a systematic search for genes involved in the mechanism of OS phagocytosis by RPE cells, herein also designated "phagocytosis-related genes," or “phagogenes,” abbreviated to "PHG." Consistent with the knowledge that AMD is a complex, multi-gene disease, and that RPE phagocytosis is a multi-step cellular process necessarily involving many different gene products, the inventors sought to identify phagocytosis-related genes based on the realization that subtle changes, such as polymorphisms, in the DNA sequences of one or more phagocytosis-related genes, or a polymorphism in a phagocytosis-related gene in combination with a polymorphism in another gene, are likely to cooperate to produce the phenotype observed in AMD.

To obtain genes of interest by differential expression, as further described in the examples below, a custom expression profiling strategy was developed, termed CHANGE (for Comparative Hybridization ANalysis of Gene Expression). The CHANGE array included approximately 10,000 genes expressed in the RPE, arrayed in panels each comprising 96 cDNAs. (See FIG. 1.) To obtain phagogenes, the CHANGE array of RPE-expressed genes was screened with pairs of "+/- OS" hybridization probes made from total RNA expressed in a phagocytic RPE cell line during OS phagocytosis *in vitro* (+ OS probe) and in control cells without feeding of OS (- OS probe). Genes in the array were selected for further analysis based upon a showing of altered (i.e., increased or decreased) expression during OS phagocytosis, evidenced by a changed hybridization signal upon hybridization with the + OS vs. – OS probes, as indicated by arrows in FIG. 1. Of the approximately 10,000 genes screened, about 60 putative phagocytosis-related genes were identified on the basis of altered gene expression detected by CHANGE. Of these, 16 genes demonstrating very pronounced change in hybridization intensity upon phagocytic challenge (i.e., screening with +/- OS probes) were randomly selected for further study and confirmation of their functional relationship to RPE phagocytosis. Table 1 provides a listing of the above-described phagogenes with subsequently confirmed association with OS phagocytosis by RPE cells. These genes are further described in Example 2, *infra*. See also FIG. 4 showing mRNA expression profiles of these genes during phagocytosis of OS by RPE cells *in vitro*.

Table 1. Human Phagocytosis-related Genes Isolated by CHANGE

NAME	CLONE NUMBER	NUCLEIC ACID SEQ ID NO.	AMINOACID SEQ ID NO(S)	IDENTITY
PHG-1	6-29	1	71-79	Unknown
PHG-2	33-25	2	80	Prostaglandin D2 synthase
PHG-3	33-74	3	81	Myelin basic protein
PHG-4	43-16	4	82-84	Unknown
PHG-5	45-88	5	85	Unknown

PHG-6	53-7	6	86	Peanut-like 2/septin 4
PHG-7	55-26	7	87	Coactosin-like 1
PHG-8	55-28	8	88	Clusterin
PHG-9	57-29	9	89	Casein kinase 1 epsilon
PHG-10	57-29	9	89	Casein kinase 1 epsilon (duplicate)
PHG-11	73-51	10	90	Ferritin heavy polypeptide 1
PHG-12	74-39	11	91	Metargidin
PHG-13	78-70a	12	92-98	Unknown
PHG-14	78-70c	13	99	Retinaldehyde binding protein 1
PHG-15	80-31	14	100	Actin gamma 1
PHG-16	91-40	15	101	Matrix metalloproteinase, membrane-associated 1 (MT1-MMP)

AMDP-Related Genes Isolated by CHANGE

In another aspect, the invention provides nucleic acid and protein sequences of genes previously unknown to be associated with AMD. To obtain AMD-related genes, the CHANGE array of 10,000 RPE-expressed genes was iteratively screened, as described above, using other pairs of "+/-" probes. The +/- probes used to identify AMD-related genes were made from total RNA extracted from the RPE/choroid of AMD-affected and unaffected human donor eyes, and from age-matched normal and affected eyes from a monkey model of AMD. Genes in the array were selected for further analysis based upon a showing of differential (i.e., increased or decreased) expression in AMD relative to aged normal control eyes. Based on the criterion of altered gene expression detected by CHANGE, approximately 200 AMD-related genes were identified.

To identify AMD-related phagogenes ("AMDP genes"), the data from the above-described two CHANGE screenings were compared, to identify a subset of RPE genes differentially expressed both in OS phagocytosis by RPE cells and in AMD. As

described above, the phagocytosis CHANGE screening yielded approximately 60 phagogenes and the putative AMD-related genes numbered approximately 200. Initial comparison of the two databases yielded a subset of 6 genes showing changed expression in both phagocytosis and AMD (Table 2). These genes are herein designated "AMD-related phagogenes" or "AMD/phagogenes," abbreviated to "AMDP."

Table 2. AMD-Related Phagogenes ("AMDP" Genes) Isolated by Iterative CHANGE Analysis

NAME	CLONE NUMBER	NUCLEIC ACID SEQ ID NO.	AMINOACID SEQ ID NO(S)	IDENTITY
AMDP-1	33-25	2	80	Prostaglandin D2 synthase
AMDP-2	37-14	16	102	SWI/SNF related/OSA-1 nuclear protein
AMDP-3	47-94	17	103-121	Unknown
AMDP-4	57-29	9	89	Casein kinase 1 epsilon
AMDP-5	73-51	10	90	Ferritin heavy polypeptide 1
AMDP-6	91-40	15	101	Matrix metalloproteinase, membrane associated 1 (MT1-MMP)

Of the above listed genes, the CHANGE hybridization analysis indicated that mRNAs for genes AMDP-1, 3, and 6 were expressed at higher levels in AMD eyes than in controls, whereas the expression levels of genes AMDP-2, 4 and 5 were lower in AMD eyes than in controls. AMDP genes are further described in Example 3, *infra*.

Nucleic Acids Encoding Phagocytosis-Related and/or AMDP-Related Gene Products and Polymorphic Variants Thereof

As described above, the invention provides nucleic acid and amino acid sequences relating to genes discovered by a differential cloning strategy (CHANGE) to exhibit altered expression during RPE phagocytosis and/or in AMD. In one aspect, the invention

provides novel purified nucleic acids (polynucleotides) isolated by this strategy. Previously unknown nucleic acids of the invention include nucleic acid sequences identified herein as PHG-1 (SEQ ID NO:1); PHG-4 (SEQ ID NO. 4); PHG-5 (SEQ ID NO: 5); PHG-13 (SEQ ID NO:12); and AMDP-3 (SEQ ID NO:17). These nucleic acids encode, respectively, polypeptides having the amino acid sequences identified herein as SEQ ID NOS:71-79; 82-84; 85; 92-98; and 103-121.

The invention also encompasses use of characterized nucleic acids and polypeptides previously unknown to be related to RPE phagocytosis and/or AMD. The relationship of the previously characterized genes to phagocytosis and AMD was discovered on the basis of changed expression during RPE phagocytosis and/or in AMD patients. Nucleic acids of the latter group include prostaglandin D2 synthase (SEQ ID NO:2), myelin basic protein (SEQ ID NO:3), peanut-like 2/septin 4 (SEQ ID NO:6); coactosin-like 1 (SEQ ID NO:7); clusterin (SEQ ID NO:8); casein kinase 1 epsilon (SEQ ID NO:9); ferritin heavy polypeptide 1 (SEQ ID NO:10); metargidin (SEQ ID NO:11); retinaldehyde binding protein 1 (SEQ ID NO:13); actin gamma 1 (SEQ ID NO:14); matrix metalloproteinase, membrane associated 1 (SEQ ID NO: 15); and SWI/SNF related/OSA-1 nuclear protein (SEQ ID NO:16).

Nucleic acid molecules of the present invention can be in the form of RNA or in the form of DNA (for example, cDNA, genomic DNA, and synthetic DNA). Preferred nucleic acid molecules of the invention are the respective native polynucleotides, including the nucleotide sequences shown herein as SEQ ID NOS:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17.

The coding sequences which encode native phagocytosis-related and/or AMDP-related genes may be identical to the those of nucleotide sequences shown in SEQ ID NOS:1-17. They may also be different coding sequences which, as a result of the redundancy or degeneracy of the genetic code, encode the same polypeptides as the polynucleotides of SEQ ID NOS:1-17. Other nucleic acid molecules within the invention are variants of SEQ ID NOS:1-17 such as those that encode fragments, analogs and derivatives of the phagocytosis-related and AMDP-related genes described herein. Such variants may be, for example, naturally occurring allelic variants of native phagocytosis-related and AMDP-related genes, homologs of native phagocytosis-related and/or

AMDP-related genes, splice variants, or non-naturally occurring variants of phagocytosis-related and/or AMDP-related genes. These variants have a nucleotide sequence that differs from the corresponding native SEQ ID NOS:1-17 in one or more bases. For example, the nucleotide sequence of such variants can feature a deletion, addition, or substitution of one or more nucleotides of native phagocytosis-related and/or AMDP-related genes.

In some applications, variant nucleic acid molecules encode polypeptides that substantially maintain a phagocytosis-related and/or AMDP-related functional activity. For other applications, variant nucleic acid molecules encode polypeptides that lack or feature a significant reduction in a phagocytosis-related and/or AMDP-related gene functional activity. Where it is desired to retain a functional activity of a native phagocytosis-related and/or AMDP-related gene, preferred variant nucleic acids feature silent or conservative nucleotide changes.

In other applications, variant phagocytosis-related and/or AMDP-related polypeptides displaying substantial changes in one or more functional activities of native phagocytosis-related and/or AMDP-related genes can be generated by making nucleotide substitutions that cause less than conservative changes in the encoded polypeptide. Examples of such nucleotide substitutions are those that cause changes in (a) the structure of the polypeptide backbone; (b) the charge or hydrophobicity of the polypeptide; or (c) the bulk of an amino acid side chain. Nucleotide substitutions generally expected to produce the greatest changes in protein properties are those that cause non-conservative changes in codons. Examples of codon changes that are likely to cause major changes in protein structure are those that cause substitution of (a) a hydrophilic residue, for example, serine or threonine, by a hydrophobic residue, for example, leucine, isoleucine, phenylalanine, valine or alanine; (b) a cysteine or proline by any other residue; (c) a residue having an electropositive side chain, for example, lysine, arginine, or histidine, by an electronegative residue, for example, glutamine or asparagine; or (d) a residue having a bulky side chain, for example, phenylalanine, by one not having a side chain, for example, glycine.

Naturally occurring allelic variants of native phagocytosis-related and/or AMDP-related genes within the invention are nucleic acids that have at least 75% (for example,

76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%) sequence identity with native phagocytosis-related and/or AMDP-related genes, and encode polypeptides having at least one functional activity in common with native phagocytosis-related and/or AMDP-related genes. Homologs of native phagocytosis-related and/or AMDP-related genes within the invention are nucleic acids isolated from non-human species that have at least 75% (for example, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%) sequence identity with native phagocytosis-related and/or AMDP-related genes, and encode polypeptides having at least one functional activity in common with native phagocytosis-related and/or AMDP-related genes.

Naturally occurring allelic variants of phagocytosis-related and/or AMDP-related genes and homologs of phagocytosis-related and/or AMDP-related genes can be isolated by screening for a native functional activity of a phagocytosis-related and/or AMDP-related gene (for example, activation of progelatinase A, in the case MT1-MMP) using techniques known in the art. The nucleotide sequence of such homologs and allelic variants can be determined by conventional DNA sequencing methods. Alternatively, public or non-proprietary nucleic acid databases can be searched to identify other nucleic acid molecules having a high percent (for example, 70, 80, 90%, 95% or more) sequence identity to a native phagocytosis-related and/or AMDP-related gene.

Non-naturally occurring variants of phagocytosis-related and/or AMDP-related genes are nucleic acids that do not occur in nature (for example, are made by the hand of man), have at least 75% (for example, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%) sequence identity with native phagocytosis-related and/or AMDP-related genes and encode polypeptides having at least one functional activity in common with native phagocytosis-related and/or AMDP-related genes. Examples of non-naturally occurring phagocytosis-related and/or AMDP-related nucleic acids are those that encode a fragment of a phagocytosis-related and/or AMDP-related protein, those that hybridize to a native phagocytosis-related and/or AMDP-related gene, or a complement of a native phagocytosis-related and/or AMDP-related genes under stringent conditions, those that

share at least 65% sequence identity with a native phagocytosis-related and/or AMDP-related gene, or a complement of a native phagocytosis-related and/or AMDP-related gene, and those that encode a phagocytosis-related and/or AMDP-related gene fusion protein.

Nucleic acids encoding fragments of phagocytosis-related and/or AMDP-related genes within the invention are those that encode, for example, 2, 5, 10, 25, 50, 100, 150, 200, 250, 300, or more amino acid residues of the respective phagocytosis-related and/or AMDP-related proteins. Shorter oligonucleotides (for example, those of 6, 12, 20, 30, 50, 100, 125, 150 or 200 bases in length) that encode or hybridize with nucleic acids that encode fragments of phagocytosis-related and/or AMDP-related genes can be used as probes, primers, or antisense molecules. Longer polynucleotides (for example, those of 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000 or more bases, such as 4000, 5000, 6000, 7000, 8000, and 9000 bases) that encode or hybridize with nucleic acids that encode fragments of phagocytosis-related and/or AMDP-related genes can be used in place of native phagocytosis-related and/or AMDP-related genes in applications where it is desired to modulate a functional activity of native phagocytosis-related and/or AMDP-related gene. Nucleic acids encoding fragments of phagocytosis-related and/or AMDP-related genes can be made by enzymatic digestion (for example, using a restriction enzyme) or chemical degradation of full length sequences of phagocytosis-related and/or AMDP-related genes, or variants thereof.

Nucleic acids that hybridize under stringent conditions to the nucleic acid of SEQ ID NOS:1, 4, 5, 12 and 17 or the complement of SEQ ID NOS:1, 4, 5, 12 and 17 are also within the invention. For example, such nucleic acids can be those that hybridize to SEQ ID NOS:1, 4, 5, 12 and 17 or the complement of SEQ ID NOS:1, 4, 5, 12 and 17 under low stringency conditions, moderate stringency conditions, or high stringency conditions. Preferred such nucleic acids are those having a nucleotide sequence that is the complement of all or a portion of SEQ ID NOS:1, 4, 5, 12 or 17. Other variants of SEQ ID NOS:1, 4, 5, 12 and 17 within the invention are polynucleotides that share at least 65% (for example, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, and 99%) sequence identity to SEQ ID NOS:1, 4, 5, 12 and 17 or the complement of SEQ ID

NOS:1, 4, 5, 12 and 17. Nucleic acids that hybridize under stringent conditions or share at least 65% sequence identity with SEQ ID NOS:1, 4, 5, 12 and 17 or the complement of SEQ ID NOS:1, 4, 5, 12 and 17 can be obtained by techniques known in the art.

Nucleic acid molecules encoding fusion proteins of phagocytosis-related and/or AMDP-related genes, for example those encoded by nucleic acids described herein as SEQ ID NOS:1-17, are also within the invention. Such nucleic acids can be made by preparing a construct (for example, an expression vector) that expresses a phagocytosis-related and/or AMDP-related fusion protein when introduced into a suitable host. For example, such a construct can be made by ligating a first polynucleotide encoding a phagocytosis-related and/or AMDP-related protein, for example MT1-MMP, fused in frame with a second polynucleotide encoding another protein such that expression of the construct in a suitable expression system yields a fusion protein.

The invention encompasses labeled nucleic acid probes capable of hybridizing to a nucleic acid encoding a phagocytosis-related and/or AMDP-related polypeptide, as described above. The nucleic acid molecules of the invention allow those skilled in the art to construct nucleotide probes for use in the detection of nucleic acid sequences of the invention in biological materials. The probe may be used in hybridization to detect a phagocytosis-related and/or AMDP-related gene. The technique generally involves contacting and incubating nucleic acids (for example mRNA molecules) obtained from a sample from a patient or other cellular source with a probe of the present invention under conditions favorable for the specific annealing of the probes to complementary sequences in the nucleic acids. After incubation, the non-annealed nucleic acids are removed, and the presence of nucleic acids that have hybridized to the probe, if any, are detected.

The detection of nucleic acid molecules of the invention may involve the amplification of specific gene sequences using an amplification method (for example PCR), followed by the analysis of the amplified molecules using techniques known to those skilled in the art. Suitable primers can be routinely designed by one of skill in the art. For example, primers may be designed using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences, Plymouth Minn.) or another appropriate program, to be about 22 to 30 nucleotides in length, to have a GC

content of about 50% or more, and to anneal to the template at temperatures of about 60^0C to 72^0C.

Hybridization and amplification techniques described herein may be used to assay qualitative and quantitative aspects of phagocytosis-related and/or AMDP-related gene expression. For example, RNA may be isolated from a cell type or tissue known to express a phagocytosis-related and/or AMDP-related gene, for example genes having SEQ ID NOS:1-17, and tested utilizing the hybridization (for example, standard Northern analyses) or PCR techniques referred to herein. The techniques may be used, for example, to detect differences in transcript size that may be due to normal or abnormal alternative splicing. The techniques may be used to detect quantitative differences between levels of full length and/or alternatively spliced transcripts detected in normal individuals relative to those individuals exhibiting symptoms of a disease. The primers and probes may be used in the above-described methods *in situ*, i.e., directly on tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies, resections or eyebank eyes. Particular uses of the probes and primers of the invention are further described in the examples below.

Genetic Screening of Phagocytosis-Related and/or AMD-Related Nucleic Acids

In another aspect, the invention provides a method for determining the risk of a subject of developing a retinal or choroidal disease or degenerative condition. As used herein, a "retinal or choroidal disease or degenerative condition" includes but is not limited to any condition of the retina or choroid of the eye which results in injury or death of photoreceptors, RPE cells or other cell types of the retina, or injury, death or abnormal proliferation of choroidal cell types including but not limited to endothelial cells, melanocytes, smooth muscle cells, fibroblasts, lymphocytes, neutrophils, eosinophils, megokaryocytes, monocytes, macrophages and mast cells.

Degenerative conditions affecting the retina and/or choroid include age-related and other maculopathies, including but not limited to age-related macular degeneration (AMD), hereditary and early onset forms of macular degeneration ("familial AMD") such as Stargardt's disease/fundus flavimaculatus, Best disease/vitelliform dystrophy, congenital diffuse drusen/Doyne's honeycomb dystrophy, pattern dystrophies, Sorsby's macular dystrophy, juxtafoveal telangiectasia, choroidal atrophy, dominant drusen,

crystalline drusen, annular macular dystrophy, occult choroidal neovascular membrane, choroideremia, idiopathic bulls-eye maculopathies, gyrate atrophy and the various forms of hereditary retinitis pigmentosa conditions. Other diseases or degenerative conditions of the retina and choroid include toxic maculopathies, for example, drug-induced maculopathies such as plaquenil toxicity, retinal disorders including retinal detachment, photic retinopathies, retinopathies induced by surgery, toxic retinopathies, retinopathy of prematurity, viral retinopathies such as CMV or HIV retinopathy related to AIDS, uveitis, ischemic retinopathies due to venous or arterial occlusion or other vascular disorders, retinopathies due to trauma or penetrating lesions of the eye, peripheral vitreoretinopathy, and cancers affecting the eye such as retinoblastoma and choriodal melanoma.

The method for determining risk involves screening a nucleic acid of a subject for the presence of polymorphisms in AMD-related or phagocytosis-related genes, wherein the presence of a polymorphism indicates that the subject is at higher risk for developing a retinal or choroidal disease or degenerative disorder than a control subject without the polymorphism. As used herein, a "normal" or "wild type" nucleotide is a base located at a particular position in a subject's DNA that is known to be the predominant base at that position in the general population. A "polymorphism," "polymorphic variant," or "polymorphic base or nucleotide," is a naturally occurring base change that occurs at lower frequency in the general population than the base representing the "wild type." A "polymorphism" as used herein can include a base change recognized as a "mutation."

A phagocytosis-related and/or AMDP-related nucleic acid of the invention, either alone or in combination with one or more other nucleic acids, may be used in hybridization, amplification and screening assays of biological samples to detect abnormalities, including point mutations, insertions, deletions, and chromosomal rearrangements. Genetic screening methods are well known in the art of molecular medicine. For example, using genomic DNA, direct sequencing, single stranded conformational polymorphism analyses, heteroduplex analysis, denaturing gradient gel electrophoresis, chemical mismatch cleavage, and oligonucleotide hybridization (including hybridization to oligonucleotides in a gene array) may be utilized. In general, a genomic DNA sample is obtained from a subject, for example from the subject's

peripheral blood, or from a biological sample prepared from donated tissue such as an eyebank eye. The DNA is used for amplification of specific gene sequences, for example a particular exonic, intronic or promoter sequence of interest. To detect the presence of polymorphisms in a subject's DNA, single strand conformation polymorphism (SSCP) analysis, heteroduplex analysis, and automated versions thereof can be used, followed by DNA sequence analysis to determine the particular base change(s). These methods are also useful for confirming reported polymorphisms, for example those available in the Human Genome Single Nucleotide Polymorphism (SNP) database.

The invention provides methods for screening a subject for polymorphic variants of genes related to RPE phagocytosis and/or AMD. In one preferred method, pairs of sense and antisense primers (amplimers) are designed based on the nucleic acid sequence of a gene of interest and are used to amplify one or more exons, introns or promoter sequences within the gene. One preferred group of genes useful for screening for mutations and polymorphisms in patients with AMD and other macular diseases includes previously unknown genes shown herein to be correlated with phagocytosis and/or AMD, the cDNA sequences of which are identified herein as SEQ ID NOS:1, 4, 5, 12, and 17. Other preferred genes, also disclosed herein to be related to phagocytosis and/or AMD, have nucleic acid (cDNA) sequences described herein as SEQ ID NOS:2, 3, 6, 7, 8, 9, 10, 11, 13, 14, 15, and 16. (See Tables 1 and 2, *supra.*) As shown herein, an exemplary gene related to AMD and phagocytosis is MT1-MMP (SEQ ID NO:15). Any amplimers suitable for amplifying an exonic, intronic or promoter sequence of a phagocytosis-related and/or AMDP-related genes disclosed herein can be designed by those of skill in the art of molecular biology and used to screen DNA samples for mutations and/or polymorphisms. As an example, specific amplimer pairs, suitable for amplification of Exons 1-10, introns 1-9 and promoter regions of the human MT1-MMP gene are disclosed in Table 3 below.

The nucleic acids of the invention can also be used for screening of multiple genes in an array. Oligonucleotides or longer fragments derived from any of the nucleic acid molecules of the invention may be used as targets in a gene array such as a microarray. The gene targets in the array can include, for example, nucleic acids derived from any combination of phagocytosis-related and/or AMDP-related genes disclosed

herein (i.e., SEQ ID NOS: 1-17) and any previously described nucleic acids, for example those previously associated with RPE phagocytosis and/or AMD, including but not limited to those derived from sequences identified herein as SEQ ID NOS:62-69. The oligonucleotide sequences included in the array can be derived from sequences positioned within an intronic, exonic or promoter sequence of the native human gene of interest. Preferably the arrays include oligonucleotide sequences encompassing all known polymorphic variants of the genes of interest. Particularly preferred custom arrays, suitable for example for sceening the DNA of patients with eye diseases such as AMD, include all known polymorphic variants of genes shown to exhibit particular polymorphic variants with increased incidence in populations of patients with AMD and related disorders, relative to control populations of normal subjects. For a listing of genes with previously reported polymorphisms or mutations correlated with AMD, see Table 5, *infra*. Accordingly, genes suitable for inclusion in a custom array of the invention useful for AMD screening, and the relevant polymorphic variants thereof showing increased incidence in AMD (in parentheses) can include, but are not limited to: MT1-MMP (P259P; D273N); ABCR (D217N; G1961E); manganese superoxide dismutase (V47A); apolipoprotein E (C130, R176C and C130R, R176); cystatin C (A25T) and paraoxonase (Q192R, L54M).

The gene arrays of the invention can be used, for example, to simultaneously monitor the expression levels of large numbers of genes, and to identify genetic variants, mutations, and polymorphisms in a plurality of genes. The information derived from the analysis of the hybridization of patient DNA samples to the array can be used, for example, to determine gene function, to understand the genetic basis of a disorder, to diagnose or predict the likelihood of developing a disorder, or to develop and monitor the activities of therapeutic agents. The preparation, use, and analysis of gene arrays, including microarrays are well known to persons skilled in the art. (See, for example, Brennan, T. M. et al. (1995) U.S. Pat. No. 5,474,796; Schena, et al. (1996) Proc. Natl. Acad. Sci. 93:10614-10619; Baldeschweiler et al. (1995), PCT Application WO95/251116; Shalon, D. et al. (I 995) PCT application WO95/35505; Heller, R. A. et al. (1997) Proc. Natl. Acad. Sci. 94:2150-2155; and Heller, M. J. et al. (1997) U.S. Pat. No. 5,605,662 and Cronin, M. et al. (2003) U.S. Patent 6,632,605.

Agents That Modulate Expression or Activity of Phagocytosis-Related and AMDP-Related Gene Products

In another aspect, the invention provides agents that modulate expression levels of mRNA or protein of phagocytosis-related and/or AMDP-related genes. Preferred genes/proteins to be targeted for down-regulation are those showing increased expression in AMD and related disorders, including, as demonstrated herein, prostaglandin D2 synthase, PD2S (respective nucleic acid and amino acid sequences: SEQ ID NOS:2 and 80), MT1-MMP (SEQ ID NOS:15 and 101) and AMDP-3 (SEQ ID NOS:17 and 103-121). Preferred genes/proteins to be targeted for up-regulation are those showing decreased expression in AMD and related disorders, including, as demonstrated herein, SWI/SNF related OSA-1 nuclear protein (SEQ ID NOS:16 and 102), casein kinase 1 epsilon (SEQ ID NOS:9 and 89) and ferritin heavy polypeptide 1(SEQ ID NOS:10 and 101).

The AMDP-related and/or phagocytosis-related mRNA or protein can be the native, i.e., "wild-type" mRNA or protein, for example native MT1-MMP. In other embodiments, a polymorphic variant of an AMD-related or phagocytosis-related gene is targeted, for example one which results in an altered function of the expressed mRNA or protein. The altered mRNA or protein is inhibited while leaving expression of the wild type mRNA or protein intact.

The inhibitory agents used for down-regulation of expression can include, for example, antisense RNA molecules, ribozymes, small interfering RNA (RNAi) molecules and triple helix structures. Preferred embodiments of such agents are directed against PD2S (SEQ ID NO:2), MT1-MMP (SEQ ID NO:15) and AMDP-3 (SEQ ID NO:17), or variants thereof. The inhibitory agents can also include antibody molecules that selectively bind to an over-expressed phagocytosis-related and/or AMDP-related protein, such as PD2S, MT1-MMP or AMDP-3.

Antisense nucleic acid molecules within the invention are those that specifically hybridize (for example bind) under cellular conditions to cellular mRNA and/or genomic DNA encoding a phagocytosis-related and/or AMDP-related protein in a manner that inhibits expression of the phagocytosis-related and/or AMDP-related protein, for example, by inhibiting transcription and/or translation. The binding may be by

conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. Methods for design of antisense molecules are well known to those of skill in the art. General approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) Biotechniques 6:958-976; Stein et al. (1988) Cancer Res 48:2659-2668; and Narayanan, R. and Aktar, S. (1996): Antisense therapy. Curr. Opin. Oncol. 8(6):509-15. As non-limiting examples, antisense oligonucleotides may be targeted to hybridize to the following regions: mRNA cap region; translation initiation site; translational termination site; transcription initiation site; transcription termination site; polyadenylation signal; 3' untranslated region; 5' untranslated region; 5' coding region; mid coding region; and 3' coding region.

An antisense construct can be delivered, for example, as an expression plasmid which when transcribed in the cell produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a phagocytosis-related and/or AMDP-related gene product. Alternatively, the antisense construct can take the form of an oligonucleotide probe generated *ex vivo* which, when introduced into a phagocytosis-related or AMDP-related gene expressing cell, causes selective inhibition of expression of the corresponding gene by hybridizing with an mRNA and/or genomic sequence coding for the phagocytosis-related or AMDP-related gene. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, for example exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see, for example, U.S. Pat. Nos. 5,176,996; 5,264,564; and 5,256,775). With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, for example, between the -10 and +10 regions of a phagocytosis-related or AMDP-related gene encoding nucleotide sequence, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to a phagocytosis-related and/or AMDP-related mRNA. The antisense oligonucleotides will bind to mRNA transcripts of the phagocytosis-related or AMDP-related gene and prevent translation. Absolute complementarity, although

preferred, is not required. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex. Oligonucleotides that are complementary to the 5' end of the message, for example, the 5' untranslated sequence up to and including the AUG initiation codon, in general work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. (See, for example, Wagner, R. (1994) Nature 372:333.) Therefore, oligonucleotides complementary to either the 5' or 3' untranslated non-coding regions of a phagocytosis-related or AMDP-related gene could be used in an antisense approach to inhibit translation of endogenous mRNA of a phagocytosis-related or AMDP-related gene. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of the mRNA of a phagocytosis-related or AMDP-related gene, antisense nucleic acids should be at least six nucleotides in length, and are preferably less than about 100 and more preferably less than about 50, 25, 17 or 10 nucleotides in length.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide, and that the nucleotide sequence of the control oligonucleotide differs from that of the antisense sequence by no more than is necessary to prevent specific hybridization to the target sequence. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein.

Antisense oligonucleotides of the invention may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouricil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-idimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Antisense oligonucleotides of the invention may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose, and may additionally include at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, for example, by use of an automated DNA synthesizer. As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988) Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared, for example, by use of controlled pore glass polymer supports (Sarin et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451).

The antisense molecules can be delivered into cells that express phagocytosis-related or AMDP-related genes *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA into cells and are well known in the art. Because it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs, a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a

strong promoter. The use of such a construct to transfect target cells in a subject preferably will result in the transcription of single-stranded RNAs that will hybridize with endogenous transcripts encoding the gene products of interest in sufficient amounts to prevent translation of the respective mRNAs. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or can become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art and are further described below. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells.

Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, and preferably human cells. Such promoters can be inducible or constitutive. Such promoters can include, but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), and the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42). Promoters useful for tissue- or cell-specific expression, for example in photoreceptors, RPE cells, or choroidal cell types such as endothelial cells or melanocytes, are also known in the art, and are further described in Example 7 below.

A ribozyme is another preferred embodiment of an agent that can down-regulate expression of a phagocytosis-related and/or AMDP-related gene product. Ribozyme molecules are designed to catalytically cleave a transcript of a gene of interest, preventing its translation into a polypeptide. (See, for example, Sarver et al. (1990) Science 247:1222-1225 and U.S. Pat. No. 5,093,246). In general, ribozymes catalyze site-specific cleavage or ligation of phosphodiester bonds in RNA. While various forms of ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy phagocytosis-related or AMDP-related mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead and hairpin ribozymes are RNA molecules that act by base pairing with complementary RNA target sequences, and carrying out cleavage reactions

at particular sites. In the case of the hammerhead, the ribozyme cleaves after UX dinucleotides, where X can be any ribonucleotide except guanosine, although the rate of cleavage is highest if X is cytosine. The catalytic efficiency is further affected by the nucleotide preceding the uridine. In practice, NUX triplets (typically GUC, CUC or UUC) are required in the target mRNA. Such targets are used to design an antisense RNA of approximately 12 or 13 nucleotides surrounding that site, but skipping the C, which does not form a conventional base pair with the ribozyme.

Synthetic hammerhead ribozymes can be engineered to selectively bind and cleave a complementary mRNA molecule, then release the fragments, repeating the process with the efficiency of a protein enzyme. This can represent a significant advantage over, for example, antisense oligonucleotides which are not catalytic, but rather are stoichiometric, forming a 1:1 complex with target sequences. The hammerhead ribozymes of the invention can be designed in a 6-4-5 stem-loop-stem configuration, or any other configuration suitable for the purpose. In general, because the chemical cleavage step is rapid and the release step is rate-limiting, speed and specificity are enhanced if the hybridizing "arms" of the ribozyme (helices I and III) are relatively short, for example, about 5 or 6 nucleotides. Suitability of the design of a particular configuration can be determined empirically, using various assays known to those of skill in the art.

The construction and production of hammerhead ribozymes is well known in the art and is described more fully, for example, in Haseloff and Gerlach (1988) Nature 334:585-591. There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequences of native phagocytosis-related or AMDP-related genes, for example, those encoded by SEQ ID NOS:1-17. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the phagocytosis-related or AMDP-related mRNA, in order to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts. Ribozymes within the invention can be delivered to a cell using a vector as described below.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been

extensively described by Thomas Cech and collaborators (see, for example, Zaug et al., (1984), Science, 224:574-578; Been and Cech, (1986), Cell, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the mRNAs specific for the peptides and proteins of interest of the current invention.

Yet another preferred agent within the invention is an RNA-mediated interference (RNAi) molecule that down-regulates expression of a phagocytosis-related and/or AMDP-related gene. The RNAi mechanism involves the use of double-stranded RNA (dsRNA) to trigger the silencing of genes highly homologous in sequence to the dsRNA. RNAi is an evolutionarily conserved phenomenon common to such diverse organisms as plants, nematodes (*Caenorhabditis elegans*), fruit flies (*Drosophila*), amphibians, and mammals. It is thought to have evolved to protect the genome against invasion by mobile genetic elements such as transposons and viruses. In a multistep process, active small interfering RNA (siRNA) molecules are generated *in vivo* through the action of an RNase III endonuclease, termed Dicer. The resulting 21-to 23-nucleotide siRNA molecules mediate degradation of the complementary homologous RNA (Zamore et al., 2000; Grishok et al., 2000).

Non-naturally occurring RNAi molecules can be synthesized by methods known in the art and used advantageously to silence the expression of genes of interest. In mammalian cells, dsRNAs longer than 30 nucleotides are known to activate an antiviral response, leading to the nonspecific degradation of RNA transcripts and a general shutdown of host cell protein translation. However, gene-specific suppression in mammalian cells can be achieved by *in vitro*-synthesized siRNAs that are about 21 nucleotides in length, these molecules being long enough to induce gene-specific suppression, but short enough to evade the host interferon response (Elbashir, S.M. et al., 2001). Those of skill in the art will recognize that computer programs are available for the design of RNAi molecules directed against specific mRNA target sequences.

Small inhibitory RNA molecules act by binding to a protein complex within the cell, termed an RNA-induced silencing complex (RISC), which contains a helicase activity and an endonuclease activity. The helicase activity unwinds the two strands of

RNA molecules, allowing the antisense strand of the siRNA to bind to the targeted RNA molecule (Zamore, 2002; Vickers et al., 2003). The endonuclease activity hydrolyzes the target RNA at the site where the antisense strand is bound.

RNAi strategies can be successfully combined with vector-based approaches to achieve synthesis in transfected cells of small RNAs from a DNA template under the control, for example, of an RNA polymerase III (Pol III) promoter. Use of Pol III provides the advantage of directing the synthesis of small, non-coding transcripts whose 3' ends are defined by termination within a stretch of 4-5 thymidines (Ts). These properties make it possible to use DNA templates to synthesize, *in vivo*, small RNAs with structural features close to those found to be required for active siRNAs synthesized *in vitro*. Using such templates, small RNAs targeting selected mRNAs of interest have been expressed in transfected cells, and shown to be able to efficiently and specifically inhibit the synthesis of the corresponding proteins (Sui et al., 2002).

For suppression of dominant gain-of-function mutations, or undesirable polymorphic variants of mRNAs of phagocytosis-related and/or AMDP-related genes which may differ from the wild type sequences by only a single base change (for example one of the AMD-associated variants of MT1-MMP, described herein), it may be desirable to selectively silence expression of the abnormal mRNA while permitting expression of the normal allele. A highly advantageous feature of the RNAi technology is the ability to selectively silence a mutation with single-nucleotide specificity. The feasibility of this approach has been demonstrated using RNAi to suppress the expression of a mutant allele of the Cu, Zn superoxide dismutase (SOD1) gene causing amyotrophic lateral sclerosis (ALS), while leaving expression of the normal allele intact (Ding et al., 2003).

The effectiveness of RNAi administration *in vivo* has been recently demonstrated in several mouse models of autoimmune hepatitis. Fas-mediated apoptosis is implicated in a broad spectrum of liver diseases. The *in vivo* silencing effect of siRNA duplexes targeting the Fas gene (also known as Tnfrsf6) encoding the Fas receptor was shown to protect mice from liver failure and fibrosis in these models. Intravenous injection of Fas siRNA specifically reduced Fas mRNA levels and expression of Fas protein in mouse hepatocytes, and the effects persisted without diminution for 10 days. In a fulminant hepatitis induced by injecting agonistic Fas-specific antibody, 82% of mice treated with

siRNA that effectively silenced Fas survived for 10 days of observation, whereas all control mice died within 3 days (Song et al., 2003). A similar RNAi-based strategy is envisioned be useful in targeting or down-regulating abnormal or over-expressed genes in AMD patients.

Alternatively, expression of phagocytosis-related and/or AMDP-related genes can be reduced by targeting deoxyribonucleotide sequences complementary to regulatory regions of the phagocytosis-related or AMDP-related gene (i.e., the phagocytosis-related or AMDP-related gene promoters and/or enhancers) to form triple helical structures that prevent transcription of the phagocytosis-related or AMDP-related gene in target cells. (See generally, Helene, C. (1991) Anticancer Drug Des. 6(6):569-84; Helene, C., et al. (1992) Ann. N.Y. Acad. Sci. 660:27-36; and Maher, L. J. (1992) Bioassays 14(12):807-15). Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription are preferably single-stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues is located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex. Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

Antisense RNA, ribozyme, RNAi and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of such molecules. These

include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art, such as for example solid phase phosphoramide chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be used.

Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone, as described above.

Other embodiments of agents that can down-regulate expression or neutralize the biological activity of the phagocytosis-related and/or AMDP-related genes of the invention are based on proteins. An example of a protein that can modulate expression and/or neutralize a biological function of a phagocytosis-related and/or AMDP-related gene product is an antibody that specifically binds a phagocytosis-related and/or AMDP-related polypeptide or peptide. Preferred polypeptides, for which mRNA levels are shown herein to be elevated in AMD, include those encoded by nucleic acids having SEQ ID NOS:2, 15 and 17, i.e., polypeptides having amino acid sequences respectively identified herein as SEQ ID NOS:80, 101, and 103-121. The antibodies of the invention can be used to interfere with the interaction of a phagocytosis-related and/or AMDP-related protein with one or more molecules that bind or otherwise interact with the phagocytosis-related and/or AMDP-related protein. For instance, an antibody directed against MT1-MMP protein is thought to neutralize the ability of this protein to activate progelatinase A. The results of a study described herein using an antibody directed against MT1-MMP showed delay of retinal degeneration in a rat model of RPE-based disease characterized by over-expression of MT1-MMP. Accordingly, inhibition of excessive production of MT1-MMP in the

interphotoreceptor matrix using an anti-MT1-MMP antibody might be used in the eyes of patients with AMD to reduce destruction of the matrix and improve phagocytosis.

The proteins encoded by the nucleic acids of the invention (for example SEQ ID NOS:1-17, or immunogenic fragments or analogs thereof, and most preferably those encoded by nucleic acids found to be up-regulated in AMD (i.e., SEQ ID NOS:2, 15 and 17) can be used to raise antibodies useful in the invention. Such proteins can be produced by purification from cells/tissues, recombinant techniques or chemical synthesis well known to those of skill in the art. Antibodies for use in the invention can include polyclonal antibodies, monoclonal antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, and molecules produced using a Fab expression library. See, for example, Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., "Monoclonal Antibodies and T Cell Hybridomas," Elsevier, N.Y., 1981; Ausubel et al., supra; U.S. Pat. Nos. 4,376,110, 4,704,692, and 4,946,778; Kosbor et al., Immunology Today 4:72, 1983; Cole et al., Proc. Natl. Acad. Sci. USA 80:2026, 1983; Cole et al., "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96, 1983; and Huse et al., Science 246:1275, 1989.

Other protein-based agents that can modulate expression or activity of a phagocytosis-related and/or AMDP-related protein include variants of phagocytosis-related and/or AMDP-related proteins that can compete with the corresponding native proteins for binding ligands, for example naturally occurring ligands that bind prostaglandin D2 synthase (SEQ ID NO:2), MT1-MMP (SEQ ID NO:15) and unknown gene AMDP-3 (SEQ ID NO:17). Such protein variants can be generated through various techniques known in the art. For example, a phagocytosis-related and/or AMDP-related protein variant can be made by mutagenesis, such as by introducing discrete point mutation(s), or by truncation. The mutation(s) can give rise to a phagocytosis-related and/or AMDP-related protein variant having substantially the same, or merely a subset of the functional activity of a native phagocytosis-related and/or AMDP-related protein. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to another molecule that interacts with a phagocytosis-related and/or AMDP-related protein. In addition, agonistic

(or superagonistic) forms of the protein may be generated that constitutively express one or more phagocytosis-related and/or AMDP-related protein functional activities. Other variants of phagocytosis-related and/or AMDP-related proteins that can be generated include those that are resistant to proteolytic cleavage, as for example, due to mutations which alter protease target sequences. Whether a change in the amino acid sequence of a peptide results in a phagocytosis-related and/or AMDP-related protein variant having one or more functional activities of a native phagocytosis-related and/or AMDP-related protein can be readily determined by testing the variant for a native phagocytosis-related and/or AMDP-related gene protein functional activity (for example, binding a receptor or other ligand, or inducing a cellular response such as phagocytosis).

Another agent that can modulate expression or activity of a phagocytosis-related and/or AMDP-related gene product is a non-peptide mimetic or a chemically modified form of a phagocytosis-related and/or AMDP-related gene product that disrupts binding of a phagocytosis-related and/or AMDP-related protein to other proteins or molecules with which the native phagocytosis-related and/or AMDP-related gene product interacts. See, for example, Freidinger et al., in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988). Examples of such molecules include azepine (for example, see Huffman et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) J. Med. Chem. 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill, 1985), beta-turn dipeptide cores (Nagai et al. (1985) Tetrahedron Lett 26:647; and Sato et al. (1986) J. Chem. Soc. Perkin. Trans. 1:1231), and beta-amino alcohols (Gordon et al. (1985) Biochem. Biophys. Res. Commun. 126:419; and Dann et al. (1986) Biochem. Biophys. Res. Commun. 134:71).

A phagocytosis-related and/or AMDP-related protein may also be chemically modified to create a protein derivative by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of phagocytosis-related and/or AMDP-related proteins can be

prepared by linking the chemical moieties to functional groups on amino acid side chains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

Yet other embodiments of agents that can modulate expression or activity of a phagocytosis-related or AMDP-related gene are small molecules. Small molecules from a wide range of chemical classes can interfere with the activity of a phagocytosis-related and/or AMDP-related protein, for example by binding to the protein and inactivating its activity, or alternatively by binding to a target of the phagocytosis-related and/or AMDP-related protein, thereby interfering with the interaction of the protein with its target. Depending upon the nature of the gene/protein of interest, inhibitory small molecules can be designed to achieve various purposes, such as 1) to occupy a binding site for a substrate or target interacting protein, 2) to bind to the phagocytosis and/or AMDP related protein so as to change its 3-dimensional conformation, thereby inhibiting its activity, or 3) to bind to a target molecule of the phago/AMDP protein, thereby inhibiting interaction of the protein with its normal target. For example, small molecule inhibitors of MT1-MMP protein (SEQ ID NO:100) are known, such as polyphenols extractable from green tea (i.e., Epigallocatechin 3-O-gallate (EGCG), (-)-epigallocatechin 3,5-di-O-gallate, and epitheaflagallin 3-O-gallate) that have potent and distinct inhibitory activity against this protein (Oku N. et al., Biol Pharm Bull. (2003) Sep;26(9):1235-8). Other classes of inhibitors of metalloproteinases in general are disclosed, for example, in Beckett, R. et al. (2001), U.S. Patent No. 6,310,084.

Gene Therapy for AMD and Other Retinal Degenerative Conditions Based on Phagocytosis-Related and AMD-Related Genes

In another aspect, the present invention provides for the delivery of natural or synthetic nucleic acids encoding phagocytosis-related and/or AMDP-related genes, or agents that modulate expression or activity of these genes. "Gene therapy" can be defined as the treatment of inherited or acquired diseases by the introduction and expression of genetic information in cells. Methods and compositions involving gene therapy vectors are described herein. Such techniques are generally known in the art and are described in methodology references such as Viral Vectors, eds. Yakov Gluzman and Stephen H. Hughes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988; Retroviruses, Cold Spring Harbor Laboratory Press, Plainview, NY, 2000; Gene

Therapy Protocols (Methods in Molecular Medicine), ed. Jeffrey R. Morgan, Humana Press, Totawa, NJ, 2001.

In the various embodiments, the nucleic acids according to the invention are incorporated into recombinant nucleic acid constructs, typically DNA constructs, capable of introduction into and replication in a host cell. Such a construct preferably is a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. For the present invention, conventional compositions and methods for preparing and using vectors and host cells can be employed, as described, for example, in Sambrook et al., *supra*, or Ausubel et al., *supra*.

Vectors useful in the practice of the invention comprise various types according to the purpose of the gene therapeutic approach. Some embodiments are vectors that include a nucleic acid encoding an agent that modulates (for example, down-regulates) expression of an AMDP-related or phagocytosis-related mRNA or protein. Other embodiments of the vectors include a wild-type or desirable polymorphic variant of a phagocytosis-related and/or AMDP-related gene of the invention. In various versions of the vectors of the former type, expression can be down-regulated by expressing, for example, an antisense RNA, ribozyme, RNAi molecule or triple helix molecule directed against an over-expressed mRNA, for example that of PD2S (SEQ ID NO:2), MT1-MMP (SEQ ID NO:15), or AMDP-3 (SEQ ID NO:17).

Other embodiments of the vectors direct expression of a desired polymorphic form of an AMDP-related or phagocytosis-related gene, either a wild-type, or a variant form. For example, in one embodiment the nucleic acid encodes a normal (wild-type) form of MT1-MMP (for example, SEQ ID NO:15). Delivery of a wild type form can be useful, for example, for subjects who do not express the normal variant, but rather are homozygous for an undesirable polymorphic form (such as a D273N missense polymorphism of MT1-MMP described herein), or are heterozygous for two different undesirable allelic forms (for example, a D273N missense polymorphism and a P259P synonomous/splice variant polymorphism).

Natural or synthetic nucleic acids according to the present invention, including cDNAs, antisense, ribozyme and RNAi molecules can be incorporated into recombinant

nucleic acid constructs, typically DNA constructs, capable of introduction into and replication in a host cell. For the present invention, conventional compositions and methods for preparing and using vectors and host cells can be employed, as described, for example, in Sambrook et al., *supra*, or Ausubel et al., *supra*. As used herein, an "expression vector" is a vector which (due to the presence of appropriate transcriptional and/or translational control sequences) is capable of expressing a DNA (encoding cDNA, antisense, ribozyme, or RNAi) molecule which has been cloned into the vector and of thereby producing an RNA or polypeptide/protein. Expression of the cloned sequences occurs when the expression vector is introduced into an appropriate host cell.

The precise nature of regulatory regions needed for gene expression may vary from organism to organism, and according to the nature of the cloned sequence and purpose for expressing the sequence in a cell, but in general these elements include a promoter which directs the initiation of RNA transcription. Such regions may include those 5'non-coding sequences involved with initiation of transcription, such as a TATA box. The promoter may be constitutive or regulatable. Constitutive promoters are those which cause an operably linked gene to be expressed essentially at all times. Regulatable promoters are those which can be activated or deactivated. Regulatable promoters include inducible promoters, which are usually "off," but which may be induced to turn "on," and "repressible" promoters, which are usually "on," but which may be turned "off." Many different regulators are known, including temperature, hormones, heavy metals, and regulatory proteins. These distinctions are not absolute; a constitutive promoter may be regulatable to some degree.

The promoter may be a "ubiquitous" promoter active in essentially all cells of the host organism, for example, the beta-actin or optomegalovirus promoters, or it may be a promoter whose expression is more or less specific to the target cell or tissue. Promoters suitable for cell-specific (for example, photoreceptor-specific, RPE-specific, and melanocyte-specific) expression in the eye, and inducible promoters used to initiate transgene expression in transgenic animals at specific ages are described in examples below.

A number of vectors suitable for stable transformation of animal cells or for the establishment of transgenic animals are known. See, for example, Pouwels et al., Cloning

Vectors: A Laboratory Manual, 1985, Supp. 1987. Typically, animal expression vectors include (1) one or more cloned animal genes under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such animal expression vectors may also contain, if desired, a promoter regulatory region (for example, a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal. Animal expression vectors within the invention preferably contain a selectable marker gene used to identify the cells that have become transformed. Suitable selectable marker genes for animal systems include genes encoding enzymes that produce antibiotic resistance (for example, those conferring resistance to hygromycin, kanamycin, bleomycin, G418, or streptomycin).

An example of a useful promoter which could be used to express a gene according to the invention is a cytomegalovirus (CMV) immediate early promoter (CMV IE) (Xu et al., Gene 272: 149-156, 2001). These promoters confer high levels of expression in most animal tissues, and are generally not dependent on the particular encoded proteins to be expressed. As an example, in most tissues of transgenic animals, the CMV IE promoter is a strong promoter. Examples of other promoters that are of use in the invention include SV40 early promoter, Rous sarcoma virus promoter, adenovirus major late promoter (MLP), Herpes Simplex Virus promoter, Mouse mammary tumor virus LTR promoter, HIV long terminal repeat (LTR) promoter, beta actin promoter (Genbank # K00790), or murine metallothionein promoter (Stratagene San Diego CA). Synthetic promoters, hybrid promoters, and the like are also useful in the invention and are known in the art.

Animal expression vectors may also include RNA processing signals such as introns, which have been shown to increase gene expression (Yu et al. (2002) 81: 155-163 and Gough et al. (2001) Immunology 103: 351-361). The location of the RNA splice sequences can influence the level of transgene expression in animals. In view of this fact, an intron may be positioned upstream or downstream of a phagocytosis-related or AMDP-related polypeptide-encoding sequence in the transgene to modulate levels of gene expression. Expression vectors within the invention may also include regulatory

control regions which are generally present in the 5' regions of animal genes. Additionally, a 3' terminator region may be included in the expression vector to increase stability of the mRNA. See, for example, Jacobson et al. (1996) Annu. Rev. Biochem. 65:693-739; and Rajagopalan et al., (1997) Prog. Nucleic Acid Res. Mol. Biol. 56:257-286.

Adenovirus vectors have been shown to be capable of highly efficient gene expression in target cells and allow for a large coding capacity of heterologous DNA. "Heterologous DNA" in this context may be defined as any nucleotide sequence or gene which is not native to the adenovirus. Methods for use of recombinant adenoviruses as gene therapy vectors are discussed, for example, in W.C. Russell, Journal of General Virology 81:2573-2604, 2000, and Bramson et al., Curr. Opin. Biotechnol. 6:590-595, 1995.

A preferred form of recombinant adenovirus is a "gutless," "high-capacity," or "helper-dependent" adenovirus vector which has all viral coding sequences deleted, and contains the viral inverted terminal repeats (ITRs), therapeutic gene (including a natural or synthetic nucleic acid encoding a phagocytosis-related or AMDP-related gene, or an agent that modulates expression of a phagocytosis-related or AMDP-related gene, up to 28-32 kb) and the viral DNA packaging sequence. Variants of such recombinant adenovirus vectors such as vectors containing tissue-specific enhancers and promoters operably linked to a natural or synthetic nucleic acids encoding a phagocytosis-related or AMDP-related gene, or agent that modulates expression of such genes are also within the invention. More than one promoter can be present in a vector. Accordingly, more than one heterologous gene can be expressed by a vector.

The viral vectors of the present invention can also include Adeno-Associated Virus (AAV) vectors. AAV exhibits a high transduction efficiency of target cells and can integrate into the host genome in a site-specific manner. Methods for use of recombinant AAV vectors are discussed, for example, in Tal, J., J. Biomed. Sci. 7:279-291, 2000 and Monahan and Samulski, Gene Therapy 7:24-30, 2000. For cell-specific targeting, a preferred AAV vector comprises a pair of AAV inverted terminal repeats which flank at least one cassette containing a promoter which directs cell-specific (for example, photoreceptor, RPE, or melanocyte) expression, operably linked to the gene of interest.

Using this vector, the DNA sequence of the AAV vector, including the ITRs, the promoter and natural or synthetic nucleic acid encoding a phagocytosis-related or AMDP-related genes, or agent that modulate expression of such a gene may be integrated into the host genome.

Another preferred vector for use in the invention is a herpes simplex virus (HSV) vector. Methods for use of HSV vectors are discussed, for example, in Cotter and Robertson, Curr. Opin. Mol. Ther. 1:633-644, 1999. HSV vectors, deleted of one or more immediate early genes (IE), are advantageously non-cytotoxic, persist in a state similar to latency in the host cell, and afford efficient host cell transduction. Recombinant HSV vectors allow for approximately 30 kb of coding capacity. A preferred HSV vector is engineered from HSV type I, deleted of the IE genes. HSV amplicon vectors may also be used according to the invention. Typically, HSV amplicon vectors are approximately 15 kb in length, possess a viral origin of replication and packaging sequences. More than one promoter can be present in the vector. Accordingly, more than one heterologous gene can be expressed by the vector. Further, the vector can comprise a sequence which encodes a signal peptide or other moiety which facilitates the secretion of the gene product from the host cell.

Viral vectors of the present invention may also include replication-defective lentiviral vectors, including HIV. Methods for use of lentiviral vectors are discussed, for example, in Vigna and Naldini, J. Gene Med. 5:308-316, 2000 and Miyoshi et al., J. Virol. 72:8150-8157, 1998. Lentiviral vectors are capable of infecting both dividing and non-dividing cells and of efficiently transducing epithelial tissues of humans. Lentiviral vectors according to the invention may be derived from human and non-human (including SIV) lentiviruses. These vectors may include the viral LTRs, primer binding site, polypurine tract, *att* sites and an encapsidation site. The lentiviral vector may be packaged into any suitable lentiviral capsid. The substitution of one particle protein by one from a different virus is referred to as "pseudotyping." The vector capsid may contain viral envelope proteins from other viruses, including Murine Leukemia Virus (MLV) or Vesicular Stomatitis Virus (VSV). The use of the VSV G-protein yields a high vector titer and results in greater stability of the vector virus particles. More than one

promoter can be present in the lentiviral vector. Accordingly, more than one heterologous gene can be expressed by the vector.

The invention also provides for use of retroviral vectors, including Murine Leukemia Virus-based vectors. Methods for use of retrovirus-based vectors are discussed, for example, in Hu and Pathak, Pharmacol. Rev. 52:493-511, 2000 and Fong et al., Crit. Rev. Ther. Drug Carrier Syst. 17:1-60, 2000. Retroviral vectors according to the invention may contain up to 8 kb of heterologous (therapeutic) DNA, in place of the viral genes. Heterologous may be defined in this context as any nucleotide sequence or gene which is not native to the retrovirus. The heterologous DNA may include a tissue- or cell-specific promoter, as described above, and a phagocytosis-related and/or AMDP-related gene. The retroviral particle may be pseudotyped, and may contain a viral envelope glycoprotein from another virus, in place of the native retroviral glycoprotein. The retroviral vector of the present invention may integrate into the genome of the host cell. More than one promoter can be present in the retroviral vector. Accordingly, more than one heterologous gene can be expressed by the vector.

To combine advantageous properties of two viral vector systems, hybrid viral vectors may be used to deliver a phagocytosis-related or AMDP-related gene or an agent that modulate expression of such a gene, to a target tissue. Standard techniques for the construction of hybrid vectors are well known to those skilled in the art. Such techniques can be found, for example, in Sambrook, et al., In Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY or any number of laboratory manuals that discuss recombinant DNA technology. Double-stranded AAV genomes in adenoviral capsids containing a combination of AAV and adenoviral ITRs may be used to transduce cells. In another variation, an AAV vector may be placed into a "gutless," "helper-dependent," or "high-capacity" adenoviral vector. Adenovirus/AAV hybrid vectors are discussed, for example, in Lieber et al., J. Virol. 73:9314-, 1999. Retroviral/adenovirus hybrid vectors are discussed, for example, in Zheng et al., Nature Biotechnol. 18:176-186, 2000. Retroviral genomes contained within an adenovirus may integrate within the host cell genome and effect stable transgene expression. More than one promoter can be present in the hybrid viral vector. Accordingly, more than one heterologous gene can be expressed by the vector.

In accordance with the present invention, other nucleotide sequence elements which facilitate expression of a phagocytosis-related or AMDP-related gene, or agent that modulate expression or activity of such a gene, and cloning of the vector are further contemplated. The presence of enhancers upstream of the promoter, or terminators downstream of the coding region, for example, can facilitate expression.

Several non-viral methods are known for introducing a phagocytosis-related and/or AMDP-related nucleic acid, or an agent that modulates expression or activity of such a nucleic acid in a cell. For a review of non-viral methods, see, for example, Nishikawa and Huang, Human Gene Ther. 12:861-870, 2001. Various techniques employing plasmid DNA for the introduction into a cell of a phagocytosis-related and/or AMDP-related nucleic acid, or an agent that modulates expression of a phagocytosis-related and/or AMDP-related nucleic acid expressed within a cell are provided for according to the invention. Such techniques are generally known in the art and are described in references such as Ilan, Y., Curr. Opin. Mol. Ther. 1:116-120 (1999); and Wolff, J.A., Neuromuscular Disord. 7:314-318 (1997).

Methods involving physical techniques for the introduction into a host cell of a phagocytosis-related and/or AMDP-related nucleic acid, or an agent that modulates expression of such a nucleic acid in a cell can be adapted for use in the present invention. Cell electropermeabilization (also termed cell electroporation) may be employed for delivery of the selected nucleic acid into cells. This technique is discussed in Preat, V., Ann. Pharm. Fr. 59:239-244 (2001), and involves the application of pulsed electric fields to cells to enhance cell permeability, resulting in exogenous polynucleotide transit across the cytoplasmic membrane. Alternatively, the particle bombardment method of gene transfer involves an Accell device (gene gun) to accelerate DNA-coated microscopic gold particles into target tissue. This methodology is described, for example, in Yang et al., Mol. Med. Today 2:476-481 (1996); and Davidson et al., Rev. Wound Repair Regen. 6:452-459 (2000).

For construction of embodiments of the invention that are transgenic animals, several standard methods are known for introduction of recombinant genetic material into oocytes for the generation of a transgenic animal. Examples of such methods include: 1) particle delivery systems (see for example, Novakovic S et al. (1999) J Exp Clin Cancer

Res 18:531-6; Tanigawa et al. (2000) Cancer Immunol Immunother 48:635-43); 2) microinjection protocols (see, for example, Krisher et al. (1994) Transgenic Res. 3: 226-231; Robinett CC and Dunaway M (1999), Modeling transcriptional regulation using microinjection into Xenopus oocytes. In: Methods: A Companion to Methods in Enzymology 17: 151-160; or Pinkert CA and Trounce IA (2002), Methods 26:348-57); (3) polyethylene glycol (PEG) procedures (see for example, Meyer O et al. (1998) J. Biol. Chem. 273:15621-7; or Park et al. (2002) Bioconj Chem, 13: 232-239); (4) liposome-mediated DNA uptake (see, for example, Hofland HEJ and Sullivan SM (1997) J. Liposome Res. 7: 187-205; or Hui SW et al. (1996) Biophys. J. 71:590-599); and (5) electroporation protocols, desribed above.

Synthetic gene transfer molecules according to the invention can be designed to form multimolecular aggregates with plasmid DNA (harboring sequences encoding a phagocytosis-related and/or AMDP-related nucleic acid, or an agent that modulates expression or activity of such a nucleic acid in a cell, operably linked to a promoter) and to bind the resulting particles to a target cell surface in such a way as to trigger endocytosis and endosomal membrane disruption. Polymeric DNA-binding cations (including polylysine, protamine, and cationized albumin) can be linked to cell-targeting ligands to trigger receptor-mediated endocytosis. Methods involving polymeric DNA-binding cations are reviewed, for example, in Guy et al., Mol. Biotechnol. 3:237-248 (1995); and Garnett, M.C., Crit. Rev. Ther. Drug Carrier Syst. 16:147-207 (1999).

Cationic amphiphiles, including lipopolyamines and cationic lipids, may provide receptor-independent gene transfer into target cells of phagocytosis-related and/or AMDP-related nucleic acids, or nucleic acids encoding an agent that modulates expression or activity of a phagocytosis-related and/or AMDP-related gene. Preformed cationic liposomes or cationic lipids may be mixed with plasmid DNA to generate cell transfecting complexes. Methods involving cationic lipid formulations are reviewed, for example, in Felgner et al., Ann. N.Y. Acad. Sci. 772:126-139 (1995); and Lasic and Templeton, Adv. Drug Delivery Rev. 20:221-266 (1996). Suitable methods can also include use of cationic liposomes as agents for introducing DNA or protein into cells. For therapeutic gene delivery, DNA may also be coupled to an amphipathic cationic peptide (Fominaya et al., J. Gene Med. 2:455-464, 2000).

Methods that involve both viral and non-viral based components may be used according to the invention. An Epstein Barr Virus (EBV) based plasmid for therapeutic gene delivery is described in Cui et al., Gene Therapy 8:1508-1513, 2001. A method involving a DNA/ligand/polycationic adjunct coupled to an adenovirus is described in Curiel, D.T., Nat. Immun. 13:141-164 (1994).

Protein transduction offers an alternative to gene therapy for the delivery of therapeutic proteins into target cells, and methods of protein transduction are within the scope of the invention. Protein transduction is the internalization of proteins into a host cell from the external environment. The internalization process relies on a protein or peptide which is able to penetrate the cell membrane. The transducing property of such a protein or peptide can be conferred upon proteins (phagocytosis-related and/or AMDP-related proteins, for example) which are expressed as fusion proteins. Commonly used protein transduction vehicles include the antennapedia peptide, the HIV TAT protein transduction domain and the herpes simplex virus VP22 protein. Such vehicles are reviewed, for example, in Ford et al., Gene Ther. 8:1-4 (2001).

Nucleic acids of the present invention may be expressed for any suitable length of time within the host cell, including transient expression and stable, long-term expression. In a preferred embodiment, a phagocytosis-related and/or AMDP-related nucleic acid, or an agent that modulates expression or activity of such a nucleic acid in a cell will be expressed in therapeutic amounts for a suitable and defined length of time. Methods of delivery that achieve either transient or long-term expression of a transgene are described herein. Episomally replicating vectors typically are maintained at intermediate to high copy number in the cell, which contributes to high levels of inserted DNA. Some vectors persist as episomes, and such vectors may behave as autonomous units replicating in the host independent of the host chromosome. DNA delivered via a plasmid or viral-based vector, including adenovirus, for example, exists in an episomal state within the host cell and is expressed in a transient manner.

Vectors according to the invention may contain nucleotide sequence elements which facilitate integration of DNA into host chromosomes. Integration is well tolerated by most transduced cells, and is preferred to ensure stability of newly introduced genetic information into a cell. Integration of a vector including a phagocytosis-related and/or

AMDP-related nucleic acid, or a nucleic acid encoding an agent that modulates expression or activity of a phagocytosis-related and/or AMDP-related gene product in a cell may occur in a random or site-specific manner. Viral-based vectors that allow for integration into the host genome include those derived from AAV, retroviruses, and some AAV/adenovirus hybrids.

The compositions comprising nucleic acid molecules (including gene therapy vectors) of the invention may be administered to a mammalian subject by any suitable technique. For example, various techniques are known using viral vectors for the introduction of a natural or synthetic nucleic acid encoding a phagocytosis-related or AMDP-related gene, or in another aspect, an agent that modulates expression or activity of a natural or synthetic nucleic acid encoding a phagocytosis-related or AMDP-related gene. Viruses are naturally evolved vehicles which efficiently deliver their genes into host cells and therefore are desirable vector systems for the delivery of therapeutic genes. Preferred viral vectors exhibit low toxicity to the host cell and produce therapeutic quantities of the natural or synthetic nucleic acid encoding a phagocytosis-related or AMDP-related gene, or agent that modulates expression or activity of such a gene, for example in a tissue-specific manner. For delivery of the vectors of the invention to the eye, various approaches are known to those of skill in the art, including intraocular injection.

Association of MT1-MMP with AMD and Other Retinal Degenerations.

Some embodiments of the invention are methods of screening, animals models of retinal degeneration and treatment methods based on matrix metalloproteinase, membrane type 1 (MT1-MMP) (SEQ ID NO:15). Among the AMDP genes listed above, one gene, i.e., MT1-MMP, (herein also designated PHG-16 and AMDP-6), was initially selected for further evaluation as a candidate target for AMD therapy. As shown in the examples below, results of various confirmatory analyses clearly demonstrated that MT1-MMP is a phagogene, as evidenced by: 1) a diurnal pattern of expression, peaking in the early morning, the time of maximal OS shedding and phagoctytosis *in vivo* (FIG. 7); 2) localization to the tips of the OS in rat and human eyes (FIGS. 8, 9); and 3) inhibition of OS phagocytosis by an antibody to MT1-MMP, both *in vitro* (FIG. 10) and *in vivo*, following subretinal injection into rat eyes (FIG. 11).

A relationship of MT1-MMP with AMD was demonstrated by: 1) correlation of a graded increase in mRNA expression with severity of AMD-related changes in human donor eyes (FIGS. 12 and 13); 2) enhanced immunolocalization of MT1-MMP antibody in the interphotoreceptor matrix in a monkey model of AMD; and 3) increased incidence of a missense polymorphism (i.e., D273N) in the catalytic domain of MT1-MMP in human macular degenerative diseases including AMD, and increased incidence in AMD and macular degeneration patients of a synonymous polymorphism in MT1-MMP (i.e., P259P). (See Table 4 in Example 5, *infra.*)

Additional studies of MT1-MMP provided evidence that overexpression of this gene is a common feature of at least one form of hereditary retinal degeneration besides AMD in which the primary etiology is in the RPE, i.e., that of the Royal College of Surgeons (RCS) rat. The RCS rat is a well known animal model of inherited retinal degeneration in which photoreceptor degeneration is due to a phagocytic defect in the RPE cells (Bok and Hall, 1971). The causative gene in this model is a mutated MERTK (D'Cruz et al. 2000). In studies described herein, MT1-MMP is shown to be overexpressed in the retina and RPE of the mutant RCS rat. Significantly, following injection of an anti-MT1-MMP antibody (2 μl volume) into the subretinal space of 7-day old RCS rats, the rate of photoreceptor degeneration relative to controls, is markedly slowed in anti-MT1-MMP antibody-injected animals observed at 30 and 60 days of age, whereas control antibodies or sham injection have no effect (FIG. 14). These results provide evidence that an agent directed against MT1-MMP protein present in the outer retina, for example within the interphotoreceptor matrix in the subretinal space, can provide a beneficial effect, such as slowing or reversing a retinal degenerative condition.

Previously recognized functions of MT1-MMP, which is expressed on invasive tumor cells, include an ability to activate progelatinase A, and to digest various ECM components (Sato et al., 1994; Cao et al., 1995; Pei and Weiss, 1996). Based on the discoveries described herein, it is now apparent that this gene provides an attractive new candidate gene to target therapeutically for AMD and other retinal and choroidal degenerative diseases.

Animal Models of AMD Based on Phagocytosis-Related and/or AMDP-Related Genes

In another aspect, the invention includes nonhuman transgenic animals (for example, mice) suitable for use as animal models of AMD and other degenerative conditions of the retina and choroid. Heretofore, testing of therapeutic compounds and treatment methods for AMD has been impeded by the lack of suitably short-lived animal models of the disease in which aging changes are practical to follow. Based on the discovery of overexpression of at least three AMD/phagogenes, i.e., PD2S (SEQ ID NO: 2), MT1-MMP (SEQ ID NO:15) and AMDP-3 (SEQ ID NO:17) in AMD eyes, and demonstration of overexpression of the MT1-MMP mRNA and protein in the retinas of humans with AMD, monkeys with AMD, and RCS rats with inherited retinal degeneration, the invention provides as preferred embodiments transgenic animals that overexpress at least one of PD2S, MT1-MMP and AMDP-3.

Some of the transgenic models are engineered to conditionally overexpress the transgene only upon addition of an exogenous stimulus, such as doxycycline. Thus, the onset of transgene expression can be controlled in these animals by administration of doxycycline. As an example, transgene expression can be triggered at a particular time of life, such as after completion of postnatal development of the retina (occurring at around 30 days of age in a mouse). The feature of inducible expression is particularly advantageous with a gene such as MT1-MMP, which if overexpressed during the embryonic or early postnatal periods might be predicted to result in developmental abnormalities in the animals. Other transgenic embodiments selectively overexpress a transgene, such as MT1-MMP, PD2S or AMDP-3 in particular cell types, for example in photoreceptors, RPE cells, or cell types of the choroid.

Yet other preferred embodiments of animal models of AMD/retinal and/or choroidal degenerations combine polymorphic variants of AMDP-related or phagocytosis-related genes, including those discovered and described herein. These models reflect the complex genetic inheritance pattern of AMD. A single genetic defect, such as a polymorphism present in MT1-MMP, may be unable to cause a disease in isolation. However, certain combinations of polymorphic variants of several genes, appropriate environmental factors, and the passage of time are likely to contribute jointly to dysfunction sufficient to tip the scale, the end result being AMD or another form of retinal, macular or choroidal degeneration. For example, other AMDP genes are likely to

cooperate with polymorphic variants of MT1-MMP to produce the full spectrum of AMD.

Accordingly, some embodiments of the transgenic animal models of AMD and other retinal and choroidal degenerations express polymorphic variants of one or more genes with involvement in AMD and/or phagocytosis by RPE cells. Various preferred embodiments are polytransgenic models expressing MT1-MMP variants, for example in combination with polymorphic variants of one or more other AMD-related genes, including those AMDP genes disclosed herein (for example, genes having the wild type cDNA sequences shown herein as SEQ ID NOS: 2, 9,10, 16, 17), and AMD-related genes having polymorphic variants previously described to be correlated with AMD (for example, SEQ ID NOS:62, 63, 64, 65, 66, 67, 68, and 69). In other preferred embodiments of the polytransgenic models, polymorphic variants of MT1-MMP are expressed in combination with polymorphic variants of other phagocytosis-related genes (for example, genes having the wild type cDNA sequences shown herein as SEQ ID NOS:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14).

EXAMPLES

The present invention is further illustrated by the following specific examples, which should not be construed as limiting the scope or content of the invention in any way.

EXAMPLE 1– Research Tools for Isolation of Phagocytosis-Related and AMD-Related Genes

Described below are research tools developed during the course of the invention, including: 1) a simple and affordable method of simultaneously gauging expression in a large number of genes by hybridization; and 2) tools for identification of phagocytosis-related genes, based on a phagocytic RPE cell line and a vital assay of phagocytosis.

CHANGE array system

Referring to FIG. 1, a macroarray technique termed Comparative Hybridization Analysis of Gene Expression (CHANGE) was developed. λgt11 cDNA libraries were constructed using techniques well known to those of skill in the art of molecular biology, from rat RPE/choroid RNA and human retinal RNA. Rat RNA used for the library was

obtained from the RPE/choroid of animals approximately 2-3 months of age, raised in cyclic light (12h light:12 hr dark), and sacrificed at various times throughout the diurnal cycle. Approximately ten thousand clones from the libraries were individually picked, amplified on plates, and transferred to blots as arrays.

Total RNA from rat and human sources was used as a global expression hybridization probe, following conversion into cDNA, amplification by PCR, and testing to confirm its usefulness for detecting expression of specific genes on the arrays.

Preliminary comparison of expression of a number of genes by CHANGE and Northern blot analysis confirmed the accuracy and demonstrated that a difference in mRNA expression as small as about 15-20% could be detected using the CHANGE method. It was apparent that the ability to readily perform iterative analysis with a combination of biologically related probes (for example, probes related on the basis of function, phenomenon, or pathology) was a very powerful aspect of this strategy.

Phagocytosis gene discovery tools

A preferred approach to identifying genes relevant to RPE phagocytosis *in vivo* is to analyze RPE gene expression in an *in vitro* system that performs the function of outer segment (OS) phagocytosis in a synchronous manner, as it occurs *in vivo*. In rodents and other mammals, shedding and phagocytosis of OS follows a circadian rhythm. Peak shedding by the photoreceptors and ingestion on a massive scale by the RPE cells is known to occur over a period of several hours beginning just before light onset (LaVail, 1976). To successfully identify phagogenes on the basis of differential expression in cultured RPE cells during the course of OS phagocytosis, it is preferable that the kinetics of the phagocytic process be uniform across the cultures, *inter alia*, to minimize "noise" from cells showing asynchronous phagocytosis with respect to their neighbors. Primary RPE cultures are generally unsuitable for this purpose, due to the marked phenotypic heterogeneity of RPE cells within primary cultures, and the corresponding heterogeneity in kinetics of phagocytosis displayed by cells of different phenotypes (McLaren, 1996).

The problem of heterogeneity can be circumvented by using an immortal RPE cell line that, like the RPE *in vivo*, demonstrates cobblestone morphology in culture, and is able to phagocytose fed OS with synchronous binding and ingestion. Methods for producing and maintaining immortal RPE cell lines from rodent and human sources are

well known in the art. An exemplary cell line exhibiting the desired phagocytic characteristics is the BPEI-1 RPE cell line (McLaren et al., 1993b). BPEI-1 cultures were shown to follow the same kinetics of OS phagocytosis as "type 1" primary RPE cells, which most closely resemble RPE *in vivo* (McLaren et al., 1993a; McLaren, 1996). Use of such cell lines for isolation of phagocytosis-related genes is preferably carried out in large-scale phagocytosis assays having sufficient cells to yield RNA amounts (about 10-30 μg) needed for both probe preparation and Northern blotting.
Accordingly, cells of a suitable RPE cell line, such as BPEI-1, are plated at high density (for example with approximately 10^6 cells per well in 6-well multi-well plates), and cultured for 1-2 days, for example in media as previously described (McLaren et al., 1993c; McLaren, 1996).

For preparation of probes for the CHANGE analysis representing specific stages of phagocytosis ("stage-specific" probes), it is advantageous to be able to follow the course of OS phagocytosis in living RPE cell cultures, to permit isolation of RNA at specific, documented, stages of the phagocytic process. To facilitate this, any suitable vital assay of OS phagocytosis can be used, for example, a double fluorescent assay previously described by McLaren et al. (1993c). Referring to FIG. 2, in this assay the lysosomes in the RPE cells are vitally stained with sulforhodamine (red fluorescence), and OS fed to the cells are prelabeled with fluorescein (FITC) (green fluorescence). The assay allows all stages of the phagocytic process (i.e., OS binding, ingestion, and digestion) to be followed by fluorescent microscopy in living cultures. Figure 3 shows different stages of synchronous binding, ingestion and intracellular processing of OS typically observed in cultures of living BPEI-1 cells at various times after feeding the cells with FITC-stained OS.

<u>Isolation of phagogenes using CHANGE</u>

To isolate phagogenes expressed at different stages of phagocytosis, stage-specific probes are prepared from total RNA extracted from the RPE cell cultures at various times (for example, 0, 1, 6, 12, 18, and 30 hours) after OS feeding, and at the same time points from control cultures not fed with OS. Following preparation of "+/-OS" phagocytosis probes by reverse transcription of the total RNA, pairs of such probes are used in a CHANGE analysis to screen a gene array, for example an array of

approximately 10,000 RPE-expressed genes as disclosed herein, to identify those genes differentially expressed during OS phagocytosis by the RPE cells. Genes showing changes in expression during OS phagocytosis are subsequently identified by DNA sequence analysis using standard techniques and compared with sequences in databases such as GenBank.

EXAMPLE 2– Isolation and Confirmation of Phagocytosis-Related Genes Expressed in RPE Cells

This example describes the isolation of genes showing changed expression during RPE phagocytosis, using the above-described methods.

From CHANGE analyses using "+/- OS" probes to screen arrays containing approximately 10,000 RPE-derived cDNAs, approximately 60 putative differentially expressed genes were initially obtained. Further detailed analyses, including confirmation of differential expression by Northern blot analysis, provided an initial subset of 16 confirmed phagocytosis-related genes selected for further investigation. Table 1 *supra* provides a listing of the identities and sequence listing notations (i.e., nucleic acids: SEQ ID NOS. 1-15 and amino acids: SEQ ID NOS:71-101) of confirmed phagogenes isolated as described herein by the CHANGE technique.

Detailed analysis of expression patterns of these genes during phagocytosis *in vitro* was examined in Northern blots of RNA extracted from BPEI cultures at various times after feeding the cells with OS. The particular stages of phagocytosis were observed in the living cells and documented photographically immediately prior to RNA extraction. As seen in FIG. 4, expression patterns of the 16 phagogenes were clustered into distinct groups that demonstrated peaks of expression at different times in the phagocytic process: i.e., early, early-mid, mid-late, and late.

EXAMPLE 3– Isolation and Confirmation of RPE-Expressed Genes Exhibiting Differential Expression in AMD

Described herein are procedures used for isolation of putative AMD genes by CHANGE, and methods for confirming their relationship to AMD.

A similar approach to that described in Example 2 utilized the CHANGE technique to identify genes related to AMD, based on the assumption that genes playing a role in the pathogenesis of AMD show changes in expression during the course of the disease. Human donor eyes were obtained from a local eye bank. Generally, eyes were accepted that were enucleated within 3 hours of death and were available for processing within 12 hours. Regardless of time of death and time elapsed until processing, the actual quality of the tissue was assessed by several criteria, including appearance on gross examination, microscopic assessment of tissue sections, and the quantity and quality of the RNA obtained, as assessed by Northern blot analysis and RT-PCR.

Referring to FIG. 5, each eye was graded microscopically for AMD-related changes, on a scale of increasing severity of AMD changes from 0 to +5, in a strip of retina/choroid, approximately 3-4 mm wide, running from periphery to periphery and passing through the optic nerve head and the macula. In assigning a grade to each eye, several morphological criteria were taken into account, including: 1) degree of thickening of Bruch's membrane; 2) number, size, and location of any drusen; 3) presence or absence of neovascularization or choridal neovascular (CNV) membranes; and 4) RPE/photoreceptor atrophy, if any. RNA, DNA, and protein were isolated from the retina and RPE/choroid of each eye.

To prepare "+ AMD" probes, total RNA was extracted from RPE/choroids of human donor eyes and pooled from multiple eyes with +3 to +5 (moderate to severe) AMD changes. Pooled RNAs from RPE/choroids of age-matched, unaffected eyes were used to prepare "- AMD" control probes. The +/- probes were used to identify differentially expressed genes by CHANGE, as described above. Approximately 200 RPE-expressed genes were initially identified that showed differential expression in subjects with AMD, compared to unaffected individuals.

To then obtain a subset of phagocytosis-related genes differentially expressed in AMD (i.e., "AMDP genes"), the results of the CHANGE screening for phagocytosis-related genes (Example 2 above) and the CHANGE screening for AMD-related genes (this example) were compared, to identify those RPE genes on the CHANGE panels demonstrating differential expression in both phagocytosis and AMD. The results of this analysis yielded an initial subset of 6 genes fitting both criteria, i.e., prostaglandin D2

synthase (SEQ ID NO:2), casein kinase epsilon 1 (SEQ ID NO:9), ferritin heavy polypeptide 1 (SEQ ID NO:10), MT1-MMP (SEQ ID NO:15), SWI/SNF related/OSA-1 nuclear protein (SEQ ID NO:16) and human unknown cDNA AMDP-3 (SEQ ID NO:17). (See also Table 2 *supra*.)

EXAMPLE 4– Isolation and Characterization of MT1-MMP as an AMD-Related and Phagocytosis-Related (AMDP) Gene

This example describes the identification of MT1-MMP (SEQ ID NO:15), an exemplary gene found by CHANGE to be differentially expressed in both phagocytosis and in AMD (i.e., an "AMD-related phagogene," or "AMDP gene"), and results of studies confirming that MT1-MMP is a phagogene and is upregulated in AMD eyes.

To identify genes related to both AMD and OS phagocytosis, the results of the two CHANGE analyses were compared as described above. Among the candidate genes differentially expressed in both screens, clone 91-40 stood out, as being a relatively new type of metalloproteinase, i.e., MT1-MMP (Sato et al., 1994) having functions that would reasonably fulfill the requirements of a gene with suspected involvement in AMD. These functions include a role in OS phagocytosis (as disclosed herein) as well as activation of progelatinase A and degradative activity against various extracellular matrix components (Sato et al., 1994; Cao et al., 1995; Pei and Weiss, 1996).

Northern blot analysis of expression of MT1-MMP in various tissues demonstrated highest levels of expression in the RPE, choroid, and retina, followed by lung and adrenal. The putative designation of MT1-MMP as a phagogene was based on its differential expression detected by CHANGE during OS phagocytosis *in vitro*. For functional confirmation, the pattern of expression of this gene was examined by Northern blot analysis in an independent assay of OS phagocytosis. Referring to FIG. 6, the result confirmed the increase in MT1-MMP expression at 13 hours after the initiation of phagocytosis, the same time of increase detected by CHANGE. The involvement of MT1-MMP in diurnally controlled OS phagocytosis *in vivo* was strongly supported by the further finding that expression of MT1-MMP mRNA, in both RPE and retina, follows a diurnal pattern with a peak at 6 AM, approximately 1-2 hours prior to the time of maximal shedding and phagocytosis of OS *in vivo* (FIG. 7).

Referring now to FIG. 8, immunofluorescent localization of MT1-MMP in the rat retina at several time points throughout the diurnal cycle demonstrated the strongest signal in the photoreceptor OS and RPE in retinas fixed at 6 AM. Immunolocalization of MT1-MMP protein in the human retina demonstrated signal in the tips of the rod, and especially cone, outer segments, consistent with activity at the interface between the photoreceptor OS membranes and the RPE apical processes, where it may be playing a role in preparing the OS tips for shedding and phagocytosis by the RPE (FIG. 9).

To obtain functional confirmation of the involvement of MT1-MMP in OS phagocytosis, an antibody against MT1-MMP (Chemicon International, Temecula, CA) was tested for its ability to inhibit OS phagocytosis by BPEI-1 cells *in vitro*. As seen in FIG. 10, the results clearly demonstrated inhibition of OS phagocytosis by this antibody, but not by an irrelevant (X-arrestin) antibody, confirming the functional requirement of MT1-MMP for the process of OS phagocytosis. Furthermore, in an *in vivo* functional assay, subretinal injection of the MT1-MMP antibody, but not X-arrestin antibody, into normal rat eyes resulted in marked structural disorganization and lengthening of the OS four days later, consistent with interference with the daily phagocytic process (FIGS. 11A, B). Thus, abundant evidence pointed to the involvement of MT1-MMP in OS phagocytosis by RPE cells.

MT1-MMP was also identified as a putative AMD gene by CHANGE on the basis of differential expression in AMD (i.e., an increase). The expression of this gene was examined independently by Northern blot analysis of RNAs from the RPE/choroid and retina of AMD-affected and normal human donor eyes. The result confirmed the increase and showed a greater increase in the retina than in the RPE (FIG. 12). As shown in FIG. 13, when a series of RNA samples from eyes with varying severity of AMD-related changes was tested, a positive correlation of increased expression of MT1-MMP in the retina was observed with increasing pathology in the eye (FIG. 13). This result strongly supported a possible role for this gene in the pathogenesis of AMD. Further, when tested in a monkey model of AMD that also showed increased expression of MT1-MMP by Northern analysis, MT1-MMP was found to be localized in the interphotoreceptor matrix (IPM) among highly disorganized OS.

Because MT1-MMP had been discovered to play a role in diurnally regulated OS phagocytosis, the inventors next tested whether the increased expression in AMD occurred at the time of maximal shedding and phagocytosis. The increase in MT1-MMP expression seen in the human eyes with AMD changes did not support this possibility, as the increase was present in eyes obtained at many different times of day after death. A plausible explanation for this result is that there may be dysregulation of MT1-MMP expression, which normally should peak only at approximately 6 AM, but in AMD may be highly active at other times as well. The functional consequence of dysregulation of MT1-MMP expression to the tightly controlled diurnal processes of OS shedding and phagocytosis could be profoundly deleterious over time.

EXAMPLE 5– Genetic Screening of MT1-MMP in Subjects with AMD and Macular Degenerative Conditions

This example describes methods for genetic analysis of MT1-MMP in AMD and macular degeneration patients and normal control populations, and results showing discovery of MT1-MMP polymorphisms correlated with macular degenerations including AMD.

Peripheral blood was collected from elderly patients affected with AMD and other macular diseases, and aged normal patients. DNA was extracted from the white blood cells. DNA was also extracted from the retina and RPE/choroid of donor eyes from a local eye bank. The degree of pathology in the donor eyes was recorded in fundus photographs and graded microscopically using the criteria described in Example 3. To enable screening for polymorphisms in MT1-MMP, all 10 exons of human MT1-MMP were determined from the published mouse gene structure (Apte et al. 1997), and amplified by PCR using human exon-specific amplimers (i.e, SEQ ID NOS:18-37) shown in Table 3 below.

Table 3. DNA Primers (Amplimers) for Amplifying Exons, Introns and Promoter Sequences of Human MT1-MMP.

<u>Exon 1:</u>

SEQ ID NO:18	9140ex1s	5'-GCCTACCGAAGACAAAGGCG-3'

SEQ ID NO:19	9140ex1a	5'-TAGAGGCTGTCCCCTAGGAG-3'
Exon 2:		
SEQ ID NO:20	9140ex2s	5'-AGAGGCACCCTATGGGCCAG-3'
SEQ ID NO:21	9140ex2a	5'-CATCTCTGGCGCTGGCATTG-3'
Exon 3:		
SEQ ID NO:22	9140ex3s	5'-GCACTGATCCCAATCCTCGC-3'
SEQ ID NO:23	9140ex3a	5'-CCCTGCATAAGCACAATGGG-3'
Exon 4:		
SEQ ID NO:24	9140ex4s	5'-GGGAAGGAGAATGTTGCCCC-3'
SEQ ID NO:25	9140ex4a	5'-GAGGAGGGAACCACCCCTAC-3'
Exon 5:		
SEQ ID NO:26:	9140ex5s	5'-GGGAGGCTGAGGGAAGGGAC-3'
SEQ ID NO:27	9140ex5a	5'-GGGGAAATGCGTAGACCAGG-3'
Exon 6:		
SEQ ID NO:28	9140ex6s	5'-CCCGCCTCCTCCTAAGTCTG-3'
SEQ ID NO:29	9140ex6a	5'-CAGCATGAGCCACCATGCCC-3'
Exon 7:		
SEQ ID NO:30	9140ex7s	5'-GAACCAGAGACCTAGGCCGC-3'
SEQ ID NO:31	9140ex7a	5'-CAGCTCCTCTAGGGAGACCC-3'
Exon 8:		
SEQ ID NO:32	9140ex8s	5'-CTAGAGCCTAAGTTGAACCC-3'
SEQ ID NO:33	9140ex8a	5'-GTGGTGGTGGTTTATGAGGG-3'
Exon 9:		
SEQ ID NO:34	9140ex9s	5'-TAGGACATGCCCATGTCCGC-3'
SEQ ID NO:35	9140ex9a	5'-TCCGCTCTTCCTCAACTCCC-3'
Exon 10:		
SEQ ID NO:36	9140ex10s	5'-CTCTTTGGGTCTTCCCTTCC-3'
SEQ ID NO:37	9140ex10s	5'-CTCTTTGGGTCTTCCCTTCC-3'
Intron 1:		
SEQ ID NO:38	9140int1s	5'CTCGGCTCGGCCCAAAGCAG 3'
SEQ ID NO:39	9140int1a	5'GTAGGTCCCCGGGAGGCAGG 3'

Intron 2:

SEQ ID NO:40	9140int2s	5'GTTTTACGGCTTGCAAGTAAC 3'
SEQ ID NO:41	9140int2a	5'CCAAACTTGTCTGGAACACC 3'

Intron 3:

SEQ ID NO:42	9140int3s	5'CCAGGGTCTCAAATGGCAAC 3'
SEQ ID NO:43	9140int3a	5'ATGTGGCATACTCGCCCACC 3'

Intron 4:

SEQ ID NO:44	9140int4s	5'CTCTGCCGAGCCTTGGACTG 3'
SEQ ID NO:45	9140int4a	5'GCATGGCCCAGCTCGTGCAC 3'

Intron 5:

SEQ ID NO:46	9140int5s	5'TGCCCGATGATGACCGCCGG 3'
SEQ ID NO:47	9140int5a	5'GGGTTGAGGGGGCATCTTGG 3'

Intron 6:

SEQ ID NO:48	9140int6s	5'CACCGTGGCCATGCTCCGAG 3'
SEQ ID NO:49	9140int6a	5'CCATCACTTGGTTATTCCTC 3'

Intron 7:

SEQ ID NO:50	9140int7s	5'CCTACGAGAGGAAGGATGGC 3'
SEQ ID NO:51	9140int7a	5'GGTTCCAGGGACGCCTCATC 3'

Intron 8:

SEQ ID NO:52	9140int8s	5'GGATGCCCAATGGAAAGACC 3'
SEQ ID NO:53	9140int8a	5'CGCTATCCACTGCCCTGAGC 3'

Intron 9:

SEQ ID NO:54	9140int9s	5'GGGATCCCTGAGTCTCCCAG 3'
SEQ ID NO:55	9140int9a	5'TGTTGAATTTCCAGTATTTG 3'

Promoter 1(-1 to -480):

SEQ ID NO:56	9140pro5s-1	5'-TATTAGTAAACTGGCCCTTC-3'
SEQ ID NO:57	9140pro3a	5'-ATCTTTCTTCTGCTTAGTCG-3'

Promoter 2 (-1 to –790):

SEQ ID NO:58	9140pro5s-2	5'-TAGAGGTGGAACTAAACCCC-3'
SEQ ID NO:57	9140pro3a	5'-ATCTTTCTTCTGCTTAGTCG-3'

As an example, exon 5 of the human MT1-MMP gene was amplified by PCR using amplimers having the nucleic acid sequences shown herein as SEQ ID NOS:26 and 27, to obtain a 285 bp wild type PCR product having the DNA sequence (SEQ ID NO:59) shown in FIG. 15. A suitable PCR amplification protocol to obtain this product was the following: 3 minutes at 95°C, 30 cycles of 1 minute at 95°C, 30 seconds at 62°C, 30 seconds at 72°C, and 5 minutes at 72°C. The 285 bp PCR product was purified by gel electrophoresis and extraction, and subjected to DNA sequencing.

Using the amplimers shown in Table 3, the MT1-MMP gene was screened for mutations and polymorphisms in DNA from patients affected with AMD and familial macular diseases, and unaffected control subjects. Screening was performed using DNA obtained from three groups of macular degeneration subjects: 1) 56 clinically documented AMD patients seen in a local clinic; 2) 22 sporadic and familial macular degeneration patients seen in ophthalmic genetics clinics; and 3) eyes from 6 eye bank donors, the eyes showing a range of +2 - +5 AMD-related changes. Clinical disease diagnoses in the familiar macular disease group of patients included familial macular dystrophy, vitelliform macular dystrophy, juxtafoveal telangiectasia, dominant drusen, crystalline drusen, annular macular dystrophy, and choroidal atrophy.

Results of screening the DNA from normal and macular degeneration-affected patients revealed a "hotspot" containing several polymorphic variants within exon 5 of MT1-MMP. A first variant was identified i.e., a synonymous polymorphism herein designated P259P, that differed between a C and G nucleotide (i.e., CC<u>C</u> Proline vs. CC<u>G</u> Proline) within codon 259 in the MT1-MMP cDNA sequence. The P259P variant base was at the 143rd base position in the 285 bp exon 5 fragment shown in FIG. 14. Referring to FIG. 14, the position of codon 259 is indicated by underlining, and the position of the P259P polymorphic variant base is indicated in boldface. The wild type DNA sequence for the human MT1-MMP exon 5 product obtained by PCR using the above-indicated primer pair is listed herein as SEQ ID NO:59, and the exon 5 sequence containing the P259P variant is listed as SEQ ID NO:60.

Analysis of potential splice donor (GT) and splice acceptor (AG) sites in the human MT1-MMP gene sequence revealed that the P259P polymorphism could give rise to a splice variant of the mRNA for MT1-MMP. Normal splicing to remove introns from

the wild type gene sequence results in a 582 amino acid full length MT1-MMP protein product (SEQ ID NO:100) including 53 amino acids encoded by exon 5 (shown herein as SEQ ID NO:121). By contrast, the P259P variant could create a new splice donor site in codon 259 that jumps to an alternate acceptor site.

Referring again to FIG. 14, a second variant was identified, herein designated D273N, which is a missense polymorphism in MT1-MMP codon 273 that differs between a G and A nucleotide (GAT Aspartic acid vs. AAT Asparagine). This polymorphism is located at the 183rd base position within the 285 bp exon 5 fragment (codon 273 underlined, and variant base boldfaced in FIG. 14). The D273N missense variant changes the wild type, charged amino acid (i.e., aspartic acid) to an uncharged amino acid (i.e., asparagine). The nucleic acid sequence of the human MT1-MMP exon 5 product obtained by PCR in subjects having the D273N polymorphism is listed herein as SEQ ID NO:61, and the corresponding predicted variant protein product from exon 5 is listed as SEQ ID NO:123.

Referring now to Table 4, results of the MT1-MMP screening analysis for the P259P synonymous polymorphism showed a frequency of 27.4% of this variant in all patients with macular disease, as opposed to 10.5% frequency in the normal population.

Table 4. Frequency of Polymorphic Variants of MT1-MMP in Macular Diseases.

Polymorphic variant	P259P Synonymous	D273N Missense	P259P or D273N
Normal subjects	10.5%	21.1%	31.6%
All macular disease subjects	27.4%	31%	58.3%
AMD	25.8%	29%	54.8%
Familial maculopathies	31.8%	36.4%	68.2%
Macular disease subjects Homozygous for variant	0%	4.8%	

A higher frequency of the D273N missense polymorphism (i.e., 31%) was also found in all macular diseases, compared to unaffected individuals (21.1%). The total number of

subjects having one of the two polymorphic variants of MT1-MMP, as opposed to the wild type base at the respective positions, was higher in the macular disease subjects (58.3%) than in the normal population (31%; p= 0.043).

Separate analysis of AMD, as opposed to familial macular diseases, revealed increased frequencies of the polymorphic variants of MT1-MMP in both AMD and familial forms of macular degenerations (Table 4). In AMD subjects, the frequency of finding one of the two polymorphic variants of MT1-MMP was 54.8%, whereas this frequency was 31.6% in the general population. In subjects with familial maculopathies, this percentage was even higher (68.2%; p= 0.029). These results strongly indicate that the presence of polymorphic variants of MT1-MMP are correlated with increased risk of developing a maculopathy, including AMD. Of note, 4.8% of the macular disease subjects, but none of the controls, were homozygous for the D273N missense polymorphism (Table 4).

EXAMPLE 6– Delay of Retinal Degeneration by an Agent that Binds MT1-MMP Polypeptide

This example describes studies demonstrating slowing of the rate of an inherited retinal degeneration in an animal (rat) model, using an agent that neutralizes MT1-MMP protein.

As described in Example 4 above, MT1-MMP was found to be overexpressed in human eyes with AMD, in a monkey model of AMD, and in the RCS rat, an animal model of an RPE-based inherited retinal degeneration. The mutant phenotype in the RCS rat, due to a mutation in the MERTK gene, is characterized by a defect in the ingestion phase of phagocytosis by the RPE cells. In separate studies, MT1-MMP was again isolated in a CHANGE analysis wherein +/- probes were prepared from retinal RNA of mutant and age-matched control RCS rats. Northern blot analysis of MT1-MMP expression in the RCS rat retina revealed that expression of MT1-MMP mRNA increased as the retinal degeneration progressed in this model. This result suggests that MT1-MMP may play a common role in the pathogenesis of multiple forms of retinal degeneration, particularly those based on a defect thought to affect primarily the RPE cells.

To test the functional involvement of MT1-MMP in the pathogenesis of the retinal degeneration in the RCS rat, a 2μl volume, (as supplied by the manufacturer), of an antibody against MT1-MMP (Chemicon, Temecula, CA), was injected subretinally into the eyes of immature (7 day) RCS rats. The course of the retinal degeneration was followed for the subsequent two months. Referring to FIG. 15, the results showed a remarkable delay of up to a 50% in the retinal degeneration, as determined by the thickness of the outer nuclear layer, observed at 1 month post-injection. Sham injection, or injection of an unrelated (i.e., X-arrestin) antibody did not produce this effect. This result further reinforces the involvement of MT1-MMP in the pathogenesis of retinal degenerations, making it an attractive therapeutic target for retinal degenerative conditions involving over-expression of MT1-MMP..

EXAMPLE 7– Animal Models of AMD That Overexpress Genes Upregulated in AMD

Studies of the pathogenesis of AMD are impeded by a lack of appropriate and practical animal models useful, for example, for testing candidate therapeutic compounds and approaches. This example describes the construction of animal models of AMD in mice that over-express genes demonstrated herein to be upregulated in AMD. In preferred embodiments, the over-expressed genes are prostaglandin D2 synthase (PD2S), MT1-MMP, and AMDP-3, comprising respective cDNA sequences identified herein as SEQ ID NOs:2, 15, and 17. In some embodiments, the genes are conditionally over-expressed, and in some versions, only in photoreceptors, RPE cells, and/or choroidal cells of the animals.

As described in examples above, overexpression or over-activity of MT1-MMP is observed in human and monkey eyes with AMD and in RCS rats with an RPE-based inherited retinal degeneration. To model the overexpression phenotype in a small laboratory rodent such as a mouse, transgenic mice overexpressing, for example, MT1-MMP are constructed. A particularly preferred embodiment is a transgenic mouse model featuring conditional overexpression of MT1-MMP in the fully-developed, and aged retinas of these animals, which advantageously avoids deleterious effects that could result from overexpression of MT1-MMP during the embryonic or early postnatal stages of development.

For constructing an animal model that conditionally overexpresses MT1-MMP, a conditional expression system can be used, such as the Tet Gene Expression System (BD Biosciences, Palo Alto, CA). Over-expression of a transgene 1000-fold or more within hours of activation with doxycycline has been reported using this system (Gossen et al., 1995). Conditional expression systems are advantageous for temporal control of gene expression, such as the overexpression of MT1-MMP, to cause the expression of the MT1-MMP transgene to begin at a selected time in the life of the animal, for example only in adults with a fully developed retina.

Transgenic mice are constructed using techniques well known to those of skill in the art, that over-express, for example, a human or a mouse MT1-MMP. Any suitable overexpression system can be used. In embodiments using the Tet system, a transgenic mouse is constructed that expresses a chimeric tetracycline-regulated transactivator rtTA (Tet-On) from a suitable promoter and a second transgene containing, for example, a human or mouse MT1-MMP cDNA connected to a Tet Response Element-silent promoter which responds to the transactivator. Administration of doxycycline to a double transgenic mouse thus constructed results in overexpression of the transgene, for example, MT1-MMP, through activation of the transactivator by doxycycline, and subsequent binding and activation of the silent promoter.

In some embodiments of transgenic mice overexpressing genes of interest such as PD2S, MT1-MMP and AMDP-3, expression of the transgene is limited to selected cell or tissue types. As is well known in the art of molecular biology, the cellular site of transgene expression can be controlled by selection of tissue- or cell-specific promoters. Accordingly, in one preferred embodiment, a transgenic model overexpresses a MT1-MMP transgene in a photoreceptor-specific manner. An exemplary promoter for this purpose is a bovine rhodopsin promoter (Zack et al., 1991), shown, for example, to be suitable for photoreceptor-specific expression of HRG4 (UNC119), in a transgenic mouse model (Kobayashi et al., 2000). Other embodiments of the transgenic mice selectively overexpress transgenes, such as MT1-MMP, PD2S or AMDP-3, in RPE cells. RPE cell-specific expression is directed, for example, by an RPE-specific promoter such as one derived from promoter regions of the genes encoding RPE65 (Boulanger et al., 2000) or cellular retinaldehyde binding protein (Kennedy et al., 1998). Yet other embodiments are

engineered to selectively express the transgenes in cell types of the choroid, for example in endothelial cells using an endothelial cell specific promoter (Cho et al., 2003), or in melanocytes and RPE cells using a promoter that drives expression of tyrosinase in pigmented cell types (Giraldo et al., 2003).

Transgenic mice are constructed by oocyte injection of a transgene-containing vector using techniques well known to those of skill in the art of molecular biology. (See, for example, Kobayashi et al.,2000). The overexpression of the selected transgene is confirmed in the appropriate tissues or cells of the transgenic animals (for example in the retina, or specifically in photoreceptors or RPE cells, or in one or more choroidal cell types) using techniques well known in the art and demonstrated in examples above, such as by Northern analysis or RT-PCR using appropriate probes or primers specific for the transgene, by Western blot analysis of proteins with an appropriate antibody, and by various immunolocalization techniques.

Pathology developing in the transgenic animals, for example in the retinas and/or RPE/choroid of these animals, is assessed by numerous known techniques, including, for example, examination of the retina by funduscopy, electroretinographic (ERG) testing, and light and electron microscopy at selected intervals throughout the lifetime of the animals, before and after activation of the transgene by administration of doxycycline, for example at 5, 10, 15, 20, 25 and 30 days of age, (with administration of doxycline at age 30 days), and at 1, 2, 5, 10, 20, 30, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400, 420, 440, 460, 480, 500, 520, 540, 560, 580, 600, 620, 640, 660, 680 and 700 days after activation with doxycycline. AMD-related pathology, such as lipofuscin accumulation, Bruch's membrane thickening, basal laminar and linear deposits, drusen formation, neovascularization, CNV membrane formation, photoreceptor/RPE atrophy or choroidal atrophy is monitored by standard techniques well known in the art.

EXAMPLE 8– Animal Models of AMD That Express Polymorphic Variants of Phagocytosis-Related and/or AMD-Related Genes

This example describes the construction of mouse models of AMD and other retinal degenerations that express one or more polymorphic variants of a phagocytosis-related or AMD-related gene.

As shown above, certain polymorphic variants of genes, including MT1-MMP, are found at higher frequency in the DNA of patients with AMD. To model the human conditions, transgenic mouse models expressing polymorphic and wild-type human genes, for example MT1-MMP, are constructed as follows. First, the baseline status of the mouse MT1-MMP gene is preferably determined. For example, it has been determined that the wild-type amino acid residue located at the position of the D273N polymorphism in the human MT1-MMP DNA sequence is conserved in the human and mouse. A polymorphism at this residue has not been demonstrated in the mouse (Mouse Genome Project). Presence of the wild type residue is confirmed in the mice used for transgenic construction, by tail biopsy, DNA isolation, and genotyping.

To construct polymorphic and control (wild type) transgenic mouse models, expressing respectively, polymorphic and wild type variants of a human gene of interest, such as MT1-MMP, cDNAs containing human polymorphic variants and wild-type MT1-MMP residues are connected to a promoter sequence suitable for driving expression of the transgene in a desired tissue or cell. For expression of the transgene throughout the body, an exemplary promoter sequence is, for example, a 385 bp human MT1-MMP promoter sequence, prepared by PCR amplification from human genomic DNA and previously determined to drive robust expression of the gene (Lohi et al., 2000). To aid identification of the transgene, in some embodiments the MT1-MMP gene is expressed as a green fluorescent protein (GFP) fusion protein, using a suitable vector construct, such as a BioSignal vector (InVitrogen, Carlsbad, CA). Other embodiments selectively express the transgene in particular tissues or cell types, driven by tissue- or cell type-specific promoters as described above.

Transgenic mice are constructed by oocyte injection of the vector using known techniques. Expression of the human polymorphic and wild type variants, for example of MT1-MMP, is confirmed in the transgenics, such as by RT-PCR with allele-specific primers and, in versions expressing GFP fusion proteins, by analysis of GFP expression, for example by fluorescence microscopy, Western blotting analysis, or immunodetection.

The transgenics are analyzed for the presence of AMD-related pathologies as described in Example 7 above.

Other embodiments of the animal models of AMD and other retinal degenerations are polytransgenic mice expressing polymorphic variants of at least two genes having a known association with AMD. In preferred embodiments, the animals express a polymorphic variant of MT1-MMP in combination with at least one other polymorphic gene variant showing a correlation with phagocytosis and/or AMD.

The polytransgenic versions of the animal models are based on the complex, multi-gene theory of AMD, which assumes that subtle mutations in a number of genes, commonly referred to as "polymorphisms," cooperate to cause, or create a susceptibility to, a disease. Accordingly, the full phenotype of AMD is likely to require the cooperation of at least two, and perhaps many, etiologic genes with the appropriate combination of polymorphisms. The causative genes may tip the scale toward development of AMD by contributing either "collectively" (for example, if related by a common function, such as involvement in the pathway of OS phagocytosis), or "cumulatively," for example, if unrelated by function, but each involved in a separate aspect of the pathogenic process leading to AMD.

A preferred embodiment of a polytransgenic model of AMD is a polytransgenic animal that co-expresses a first polymorphic variant of MT1-MMP and at least a second polymorphic variant of at least one other phagocytosis-related and/or AMD-related gene. Any other second or more gene showing a polymorphic variant correlated with AMD can be combined with any polymorphic variant of MT1-MMP. Genes presently reported to have variants correlated with AMD are listed in Table 5.

Table 5. Genes with Reported Polymorphisms or Mutations Correlated with AMD

GENE	NUCLEIC ACID SEQ ID NO:	AMINO ACID SEQ ID NO:	REFERENCE
ABCR	62	124	Allikmets et al.,1997
Apolipoprotein E	63	125	Klaver et al. 1998; Simonelli et al. 2001
C-C chemokine receptor-2	64	126	Ambati et al. 2003
Cystatin C	65	127	Zurdel et al. 2002
Hemicentin/FIBL-6	66	128	Schultz et al. 2003
Manganese superoxide	67	129	Kimura et al. 2000

dismutase			
C-C chemokine ligand/monocyte chemoattractant protein 1	68	130	Ambati et al. 2003
Paraoxonase	69	131	Ikeda et al. 2001

Accordingly, in one form of the preferred embodiments, a polymorphic form of MT1-MMP is combined with a polymorphic form of at least one other gene, including ABCR, apolipoprotein E, C-C chemokine receptor-2, cystatin C, hemicentin/FIBL-6, manganese superoxide dismutase, C-C chemokine ligand/monocyte chemoattractant protein 1, and paraoxonase.

Similarly, a polytransgenic model reflecting the "collective" etiology theory of AMD combines polymorphic variants of genes with known involvement in the mechanism of an important function (for example OS phagocytosis) with polymorphic variants of MT1-MMP (a demonstrated phagocytosis-related gene as disclosed herein; wild type cDNA sequence: SEQ ID NO:15; wild type amino acid sequence: SEQ ID NO:100). Such genes include, for example, polymorphic variants of phagocytosis-related genes PHG-1 to PHG-15 (SEQ ID NOS:1-14) and AMDP-2 and 3 (SEQ ID NOS:16 and 17), disclosed herein (see Tables 1 and 2, *supra*).

For construction of the models, DNA containing the reported polymorphic variant(s) of a selected gene is first isolated using appropriate amplimers from DNA of patients with AMD and unaffected, age-matched individuals (for example, as described for MT1-MMP in Example 5 above), and is used to confirm the presence of the reported polymorphisms , for example, in ABCR (i.e., D2177N, G1961E); manganese superoxide dismutase (i.e., V47A); apolipoprotein E (i.e., epsilon2); cystatin C (i.e., A and B allele, including the Ala to Thr change); and paraoxonase (i.e., Q192R, L54M). Genotyping and mutational analyses are carried out using established methods (see for example, Mashima et al., 1994). The association of the polymorphism with AMD is confirmed and the statistical significance of any detected correlations with AMD is determined, for example by a chi-square test. For those polymorphic genes showing an association with AMD, their co-occurrence with a polymorphism in MT1-MMP is then confirmed.

Trangenic mice expressing a polymorphic variant of a selected gene, for example AMDP-3, are first constructed as generally described above. To construct polytransgenic models, transgenic mice expressing a polymorphic variant of the first gene of interest, for example, MT1-MMP, are crossed with transgenic mice expressing a polymorphic variant of a second phagocytosis/AMD-related gene of interest, such as AMDP-3. Expression of the various transgenes is confirmed in tissues of interest, for example the retina, RPE or choroid, by standard techniques known in the art, such as allele-specific RT-PCR of RNA and/or immunodetection of the polymorphic transgene protein of interest, for example by using antibodies specific for a particular polymorphic form of the protein. Alternatively, in embodiments in which a specific tag protein sequence is attached to the transgene protein, identification of the tag sequence is used to facilitate identification of the transgenic polymorphic variant protein and to distinguish it from the wild-type form. The polytransgenic mouse is analyzed for evidence of AMD-related changes as described above.

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